REMARKS

This paper is a Response to the Office Action mailed December 5, 2011. Claims 28 to 32, 34 to 45, and 48 to 50 are under consideration.

Regarding the Claim Amendments

The claim amendments were made to address informalities. In particular, the amendments were made to address the claim objections and indefiniteness rejection. Thus, as the claim amendments were made to address informalities, no new matter has been added and entry thereof is respectfully requested.

Regarding the Claim Objections

Claims 28 and 43 have been objected to due to for informalities. Claims 28 and 43 have been amended to correct the informalities. Accordingly, the objection is moot.

Rejection under 35 U.S.C. 112, second paragraph

The rejection of claims 28 and 50 under 35 U.S.C. §112, second paragraph, as allegedly indefinite, is respectfully traversed. The grounds for rejection are set forth in the Office Action at pages 3 to 4.

Claim 28 has been amended to insert the indefinite article "a" before the first instance of TNFR. Because a TNFR can be TNFR1 or TNFR2 as disclosed in the specification, claim 28 is clear and definite. Claim 50 has been amended to refer to the antibody fragment as a human antibody fragment. Consequently, Applicants respectfully request that the rejection under 35 U.S. C. §112, second paragraph, be withdrawn.

Rejections under 35 U.S.C. 112, first paragraph, enablement

The rejection of claims 28-32, 34-45 and 48-50 under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement is respectfully traversed. The grounds for rejection are set forth in the Office Action at pages 4 to 9.

The claims are adequately enabled. It appears that the grounds for rejection relate to the belief that producing variants and fragments of TNF monomer that retain TNFR binding activity would require undue experimentation.

Applicants respectfully point out that the relationship between structure of TNF monomer and TNFR binding function was known in the art at the time of the invention. Furthermore, many TNF variants and fragments having TNFR binding function were known in the art at the time of the invention.

Applicants first respectfully point out that the Idriss et al. reference (Microsc. Res. Tech. 50:184 (2000)) cited by the Patent Office supports Applicants' position. In particular, as acknowledged by the Examiner Idriss et al. mention various changes that affect TNFR binding of TNF. In particular Arg31 substituted with Asp had reduced TNFR2 binding. Thus, in view of Idriss et al. one of skill in the art would know that this TNF variant would be more selective for binding to TNFR1, and could therefore make the described TNF variant for selectively binding TNFR2 without undue experimentation a.

Idriss et al. also mention another TNF variant (M3S) that had 11-71 fold less affinity for TNFR1 and TNFR2. Thus, in view of Idriss et al. one of skill in the art would know that if they modified TNF as in variant M3S that this would reduce TNFR binding.

As further evidence that the knowledge and skill in the art at the time of the invention was such that the relationship between structure of TNF monomer and TNFR binding function was known and one of skill in the art could produced TNF variants and fragments that bind TNFR without undue experimentation, submitted herewith as Exhibit A is US Patent 6,306,820, issued on October 23, 2001, to an application filed on June 4, 1999, almost 5 years prior to the March 26, 2004 priority date of the subject application. Notably, issued claims 1 and 5 recite a "TNF binding protein." Issued claims 3 and 8 each recite "sTNFR-1, sTNFR-II, sTNFR fragments." In view of the fact that these claims issued on an application filed almost 5 years prior to the March 26, 2004 priority date of the subject application, clearly the USPTO considered the genus of TNF binding proteins and TNF "fragments" enabled under 35 U.S.C. §112, first paragraph, as of the June 4, 1999 filing date.

Applicants also respectfully direct the Examiner's attention to Exhibits B-E submitted herewith. Each of Exhibits B-E, published prior to the March 26, 2004 priority date, describe variants of TNF that either have increased binding, similar binding, or reduced binding to TNFR compared to wild type TNF.

Exhibit B (Nakamura et al., Int. J. Cancer 48:744 (1991)) describes TNF mutant 471 in which the 7 N-terminal amino acids were deleted, and amino acid positions 8-10 were replaced with an Arg-Lys-Arg sequence. The authors reported that TNF mutant 471 had a higher binding activity for TNF receptors on cells than wild type TNF. Thus, in view of Nakamura et al. one of skill in the art would know how to make the described variant TNF having higher binding affinity for TNF receptors without undue experimentation.

Exhibit C (Ostade et al., Eur. J. Bioch 220:771 (1994)) describes structural regions important for activity of human TNF, such as the three loops defined by positions 30-36, 84-88 and 138-150. Mutations in these regions were typically found to reduce affinity for TNFR with a few exceptions (abstract), notably mutants R32W, E146K and D143N resulted in altering binding preference to two TNFRs studied. Thus, in view of Ostade et al. one of skill in the art would know that the three loops defined by positions 30-36, 84-88 and 138-150 of TNF are important for TNFR binding affinity and/or specificity, and would also be able to produce TNF variants having altered binding affinity and/or specificity for TNF receptors without undue experimentation.

Exhibit D (Xi et al., Biochem. Mol. Biol. Intn'l. 38:1183 (1996)) describes a mutant TNF, denoted TNF-B that has 4 amino acids deleted from the N-terminus. TNF-B was reported to have greater cytotoxicity than wild type TNF (abstract). Thus, in view of Xi et al. one of skill in the art would know how to make the described TNF variant that would bind to TNF receptors without undue experimentation.

Exhibit E (Shin et al., Biochem. Mol. Biol. Intn'l. 44:1075 (1996)) describes a mutant TNF, denoted M3 in which Ser and Tyr at residues 52 and 56 were substituted with Ile and Phe, respectively, and in which 7 amino acids from the amino terminus were deleted. The authors reported that the M3 mutant had a binding affinity for TNF receptors more than 1-fold greater than that of wild type TNF (abstract). Thus, in view of Shin et al. one of skill in the art would know how to make yet another TNF variant with higher binding affinity for TNF receptors without undue experimentation.

In sum, in view of the substantial understanding of the relationship between the structure (sequence) of TNF monomer and TNFR binding function at the time of the invention, and that many TNF variants having greater, similar or reduced TNFR binding function, as well as an altered binding preference for TNFRs were known at the time of the

invention as corroborated by Exhibits B-E, the skilled artisan would be able to make a number of different TNF variants without undue experimentation. Furthermore, in view of the fact that the USPTO allowed claims covering TNF variants and fragments on an application filed on the June 4, 1999, clearly TNF variants and fragment were considered enabled as of June 4, 1999. Consequently, claims 28-32, 34-45 and 48-50 are adequately enabled and Applicants respectfully request withdrawal of the rejection under 35 U.S.C. §112, first paragraph.

Rejections under 35 U.S.C. 112, first paragraph, written description

The rejection of claims 28-32, 34, 26, 37, 39, and 40 under 35 U.S.C. §112, first paragraph, as allegedly lacking written description is respectfully traversed. The grounds for rejection are set forth in the Office Action at pages 9 to 12.

The claims are adequately described under 35 U.S.C. §112, first paragraph. It appears that the grounds for rejection relate to the belief that TNF monomer variants and fragments that retain TNFR binding activity are insufficiently described and would require undue experimentation.

As set forth by the court, written description does not require examples or an actual reduction to practice. *Ariad Pharma, Inc. v. Eli Lilly & Co.,* 598 F.3d 1336, 1352 (Fed. Cir. 2010). Instead, written description is satisfied when one of skill in the art can "visualize or recognize" the claimed subject matter based on the specification. *Regents of the Univ. of California v. Eli Lilly & Co.,* 119 F.3d 1559, 1568 (Fed. Cir. 1997).

As discussed above, US Patent 6,306,820, issued on October 23, 2001 to an application filed June 4, 1999, almost 5 years prior to the March 26, 2004 priority date of the subject application. Notably, issued claims 1 and 5 recite a "TNF binding protein," and issued claims 3 and 8 each recite "sTNFR-1, sTNFR-II, sTNFR fragments." In view of the fact that these broad claims issued on an application filed almost 5 years prior to the March 26, 2004 priority date of the subject application, clearly the USPTO considered the genus of TNF binding proteins and fragments to meet the description requirement under 35 U.S.C. §112, first paragraph as June 4, 1999.

Furthermore, in view of the substantial understanding of the relationship between structure of TNF monomer and TNFR binding function at the time of the invention, and that

numerous TNF variants having TNFR binding function were known at the time of the invention as corroborated by Exhibits B-E, one of skill in the art would know of a large number of TNF variants having TNFR binding activity. Consequently, as one of skill in the art would be apprised of a large number of TNF variants having TNFR binding activity, claims 28-32, 34-45 and 48-50 are adequately described and the rejection under 35 U.S.C. §112, first paragraph must be withdrawn.

Rejections under 35 U.S.C. 103(a)

The rejection of claims 28-32, 34, 36, 37, 39, 40 under 35 U.S.C. §103(a), as allegedly being unpatentable over Lentz (WO 99/61085) and Klysner et al. (US 2003/0185845) is respectfully traversed. The grounds for rejection are set forth in the Office Action at pages 12 to 14.

Claims 28-32, 34, 36, 37, 39, 40 would not have been obvious in view of Lentz (WO 99/61085) alone, or in combination with Klysner et al. (US 2003/0185845).

Applicants respectfully point out that Lentz (WO 99/61085) describes dialysis or filter for treatment of cancers to remove compounds less than 120 KDa (page 2,line 26, to page 3, line 2). At most, Lentz mention prophetically removing TNFRs by binding to a cytokine (page 3, lines 2-10). Lentz fail to describe making any TNF-based material suitable for removing TNFRs, let alone successful removal of TNFRs.

Klysner describes making immunogenic analogs of various cytokine multimers (see, abstract, etc.). Such immunogenic analogs are proposed to break autologous tolerance and are used to stimulate immune responses as in vaccination (see, e.g., para [023]) One such cytokine is TNF.

The immunogenic analogs described by Klysner are designed to elicit an immune response, and used analogously to vaccines. Thus, one of skill in the art would not have been motivated to use immunogenic analogs of TNF that elicit immune responses, which are essentially foreign antigens, in the context of depleting or removing a cytokine receptor, such as TNFR, from blood or plasma. Consequently, there would not have been any reason or motivation for one of skill in the art to combine Klysner with Lentz at the time of the invention.

Furthermore, TNF monomers form a non-covalently linked trimer of TNF monomers, and this TNF trimer in turn binds to TNF receptor (TNFR). Thus, if one of skill in the art were to produce a surface or particle coupled to TNF monomers according to Lentz's prophecy, such a material would contain immobilized TNF monomers, but also a substantial number of TNF monomers that were not immobilized due to covalent binding of TNF monomers to the immobilized TNF monomers (for every immobilized TNF monomer, there would be 2-noncovalently bound TNF monomers). If such a material were made and used for depletion or removal of TNFRs, there would be substantial amounts of the non-covalently bound TNF monomers leached into the blood of blood fraction during extracorporeal depletion or removal of TNFRs. Because TNF is highly toxic, this would lead to substantial amounts of TNF monomers in the blood of blood fraction, which would be too dangerous to use in a clinical setting and would therefore never be used for extracorporeal depletion or removal of TNFRs.

In support of Applicants position that TNF monomers would substantially leach if used in the manner that Lentz at most prophetically mention, submitted herewith as Exhibit F is a study showing the leach rates of scTNF-alpha coupled to surface or particle compared to leach rates of TNF-alpha coupled to surface or particle. As illustrated, leach rates for TNF-alpha were substantially greater (approximately 50-fold greater) than for scTNF-alpha. Thus, clearly TNF monomers used in the manner prophetically mentioned by Lentz would lead to substantial TNF leaching into blood or blood fraction which would be extremely dangerous for clinical use and would therefore never be used for extracorporeal depletion or removal of TNFR.

Moreover, combining Klysner with Lentz would actually result in an even worse situation if used for extracorporeal depletion or removal of TNFR. In particular, combining Klysner with Lentz would lead to the leaching of highly toxic and immunogenic TNF analogs into the blood or blood fraction. Thus, even if for the sake of argument one of skill in the art would have combined Klysner with Lentz, the resulting column would leach highly toxic and immunogenic TNFs, which are essentially foreign antigens, into the blood or blood fraction, which would be even more dangerous in a clinical setting and would therefore never be used for extracorporeal depletion or removal of TNFR.

In sum, one of skill in the art would not have had any motivation to combine Klysner with Lentz at the time of the invention at least because: 1) if one were to use surface or particle coupled TNF monomers to remove TNFR according to Lentz this would lead to introducing substantial amounts of TNF monomers into the blood of blood fraction as corroborated by the data in Exhibit F, and would be too dangerous to use for extracorporeal depletion or removal of TNFR; 2) immunogenic TNF analogs of Klysner function as foreign antigens designed to elicit an immune response, and one of skill in the art would not have had a motivation to use such immunogenic TNF analogs in the context of removing TNFR according to Lentz; and 3) even if combined the result would lead to introducing highly toxic and immunogenic TNF monomer analogs into the blood or blood fraction and would be too dangerous to use for extracorporeal depletion or removal of TNFR. Consequently, claims 28-32, 34, 36, 37, 39, 40 would not have been obvious in view of Lentz (WO 99/61085) alone, or in combination with Klysner et al. (US 2003/0185845) and the rejection under 35 U.S.C. §103(a) must be withdrawn.

CONCLUSION

In summary, for the reasons set forth herein, Applicants maintain that the amended and new claims clearly and patentably define the invention, respectfully request that the Examiner reconsider the various grounds set forth in the Office Action, and respectfully request the allowance of the claims which are now pending.

If the Examiner would like to discuss any issues, Applicants' representatives can be reached at (858) 509-4065. Please charge any fees associated with the submission of this paper to Deposit Account Number 33975. The Commissioner for Patents is also authorized to credit any over payments to the above-referenced Deposit Account.

Respectfully submitted,

PILLSBURY WINTHROP SHAW PITTMAN LLP

By _____
Robert M. Bedgood
Reg. No. 43488
Tel. No. (858) 509-4065

Date: May 4, 2012
P.O. Box 10500
McLean, VA 22102
Customer No.: 27500

US006306820B1

(12) **United States Patent**
Bendele et al.

(10) **Patent No.: US 6,306,820 B1**
(45) **Date of Patent: Oct. 23, 2001**

(54) **COMBINATION THERAPY USING A TNF BINDING PROTEIN FOR TREATING TNF-MEDIATED DISEASES**

(75) Inventors: **Alison M. Bendele**, Nederland; **Regina M. Sennello**, Boulder, both of CO (US); **Carl K. Edwards III**, Thousand Oaks, CA (US)

(73) Assignee: **Amgen Inc.**, Thousand Oaks, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/326,394**

(22) Filed: **Jun. 4, 1999**

Related U.S. Application Data

(63) Continuation of application No. PCT/US97/22733, filed on Dec. 8, 1997.
(60) Provisional application No. 60/032,587, filed on Dec. 6, 1996, provisional application No. 60/036,355, filed on Jan. 23, 1997, provisional application No. 60/039,315, filed on Feb. 7, 1997, and provisional application No. 60/052,023, filed on Jul. 9, 1997.

(51) **Int. Cl.**[7] **A61K 38/17**

(52) **U.S. Cl.** **514/2**; 530/350

(58) **Field of Search** 514/2; 530/350

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,179,337	12/1979	Davis et al. .	
4,289,690	9/1981	Pestka et al. .	
4,766,106	8/1988	Katre et al. .	
4,847,325	7/1989	Shadle et al. .	
4,917,888	4/1990	Katre et al. .	
4,935,233	6/1990	Bell et al. .	
4,959,314	9/1990	Mark et al. .	
5,116,964	5/1992	Capon et al. .	
5,122,614	6/1992	Zalipsky .	
5,136,021	8/1992	Dembinski et al. .	
5,153,265	10/1992	Shadle et al. .	
5,166,322	11/1992	Shaw et al. .	
5,211,945	5/1993	Wallach et al. .	
5,252,714	10/1993	Harris et al. .	
5,359,037	10/1994	Wallach et al. .	
5,395,760	3/1995	Smith et al. .	
5,446,090	8/1995	Harris .	
5,478,925	12/1995	Wallach et al. .	
5,512,544	4/1996	Wallach et al. .	
5,605,690	2/1997	Jacobs et al. .	
5,610,279	3/1997	Brockhaus et al. .	
5,633,145	5/1997	Feldmann et al. .	
5,695,953	12/1997	Wallach et al. .	
5,712,155	1/1998	Smith et al. .	
5,739,208	4/1998	Harris .	
5,747,639	5/1998	Seely .	
5,808,029	9/1998	Brockhaus et al. .	
5,811,261	9/1998	Wallach et al. .	
5,843,791	12/1998	Hauptmann et al. .	
5,863,786	1/1999	Feldmann et al. .	
6,096,728 *	8/2000	Collins et al.	514/62

FOREIGN PATENT DOCUMENTS

2003743	5/1990	(CA) .
0 259 863 A2	3/1988	(EP) .
0 308 378 A2	3/1989	(EP) .
0 334 165 A2	9/1989	(EP) .
0 398 327 A1	11/1990	(EP) .
0 412 486 A1	2/1991	(EP) .
0 418 014 A1	3/1991	(EP) .
0 422 339 A1	4/1991	(EP) .
0 433 900 A1	6/1991	(EP) .
0 512 528 A2	11/1992	(EP) .
0 526 905 A2	2/1993	(EP) .
2 218 101 A	11/1989	(GB) .
2 246 569 A	2/1992	(GB) .
90339	5/1989	(IL) .

(List continued on next page.)

OTHER PUBLICATIONS

Abuchowski et al., "Effect of Covalent Attachment of Polyethylene Glycol on Immunogenicity and Circulating Life of Bovine Liver Catalase", *Journal of Biological Chemistry,* 252(11):3582–3586 (1977).

Aggarwal, et al., "Characterization of Receptors for Human Tumour Necrosis Factor and Their Regulation by γ–Interferon", *Nature,* 318:665–667 (1985).

Akerblom et al., "Preparation and Characterization of Conjugates of Monoclonal Antibodies and Staphylococcal Enterotoxin A Using a New Hydrophilic Cross–Linker", *Bioconjugate Chem.,* 4:455–466 (1993).

Ashkenazi et al., "Protection Against Endotoxic Shock by a Tumor Necrosis Factor Receptor Immunoadhesin", *PNAS* 88:10535–10539 (1991).

Baglioni et al., "Binding of Human Tumor Necrosis Factor to High Affinity Receptors on HeLa and Lymphoblastoid Cells Sensitive to Growth Inhibition", *Journal of Biological Chemistry,* 260(25):13395–13397 (1985).

Barrera P. et al., "Circulating Soluble Tumor Necrosis Factor Receptors, Interleukin–2 Receptors, Tumor Necrosis Factor Alpha, and Interleukin–6 Levels in Rheumatoid Arthritis. Longitudinal Evaluation During Methotrexate and Azathioprine Therapy.", Arthritis & Rheumatism, 36(8): 1070–1079 (1993).

Bazzoni et al., "The Tumor Necrosis Factor Ligand and Receptor Families", *Seminars in Medicine of the Beth Israel Hospital,* Boston, 334(26):1717–1725 (1996).

(List continued on next page.)

Primary Examiner—Lorraine Spector
Assistant Examiner—Eileen B. O'Hara
(74) *Attorney, Agent, or Firm*—Thomas D. Zindrick; Steven M. Odre; Ron K. Levy

(57) **ABSTRACT**

The invention relates to methods for treating or preventing acute and/or septic shock. The method comprises administering to patients in need thereof therapeutically effective amounts of a TNF binding protein and a Fas antigen. In a preferred embodiment, the TNF binding protein is sTNFR-I or sTNFR-II. The invention also relates to pharmaceutical compositions containing a TNF binding protein and a Fas antigen useful in such methods.

8 Claims, 8 Drawing Sheets

FOREIGN PATENT DOCUMENTS

WO 90/12874 A2	11/1990	(WO) .
WO 90/13575 A1	11/1990	(WO) .
WO 91/03553 A1	3/1991	(WO) .
WO 92/01002 A1	1/1992	(WO) .
WO 92/01472 A1	2/1992	(WO) .
WO 92/07076 A1	4/1992	(WO) .
WO 92 07585 A	5/1992	(WO) .
WO 92/13095 A1	8/1992	(WO) .
WO 92/16221 A1	10/1992	(WO) .
WO 93/01498 A1	1/1993	(WO) .
WO 94/06476 A1	3/1994	(WO) .
WO 95/13312 A1	5/1995	(WO) .
WO 95/34326 A1	12/1995	(WO) .
WO 96/20729 A	7/1996	(WO) .
WO 98 05357	2/1998	(WO) .

OTHER PUBLICATIONS

Beutler et al., "Passive Immunization against Cachectin/Tumor Necrosis Factor Protects Mice from Lethal Effect of Endotoxin", *Science,* 229:869–871 (1985).

Beutler and Cerami, "The Biology of Cachectin/TNF—A Primary Mediator of the Host Response", *Ann. Rev. Immunol.,* 7:625–655 (1989).

Bologna, C. et al., "Association Des Traitements De Fond Dans La Polyarthrite Rheumatoid", Presse Medicale, 25(19):876–878 (1996) Abstract.

Borigini et al., "Combination Therapy", *Bailliere S Clinical Rheumatology,* 9(4):689–710 (1995).

Brockhaus et al., "Identification of Two Types of Tumor Necrosis Factor Receptors on Human Cell Lines by Monoclonal Antibodies," *Proc. Natl. Acad. Sci. USA,* 87:3127–3131 (1990).

Corcoran et al., "Characterization of Ligand Binding by the Human p55 Tumour–Necrosis–Factor Receptor Involvement of Individual Cysteine–Rich Repeats," *Eur. J. Biochem.,* 223: 831–840 (1994).

Creasey et al., "A High Molecular Weight Component of the Human Tumor Necrosis Factor Receptor is Associated with Cytotoxicity," *Proc. Natl. Acad. Sci. USA,* 84:3293–3297 (1987).

Davis et al., "Soluble, Nonantigenic Polyethylene Glycol–Bound Enzymes", *Biomedical Polymers: Polymeric Materials and Pharmaceuticals for Biomedical Use,* Goldberg et al. (Ed.). published by Academic Press (NY), pp. 441–452 (1980).

Dayer et al., "Purification and Characterization of Human Tumor Necrosis Factor α Inhibitor," *Chemical Abstracts,* 113(38760n):454 (1990).

Dayer et al., "Interleukin–1, Tumor Necrosis Factor and Their Specific Inhibitors," *European Cytokine Network,* 5(6):563–571 (1994).

Elliott et al., "Randomised Double–blind Comparison of Chimeric Monoclonal Antibody to Tumour Necrosis Factor α (cA2) versus Placebo in Rheumatoid Arthritis", *Lancet,* 344:1105–1110 (1994).

Engelmann et al., "A Tumor Necrosis Factor–Binding Protein Purified to Homogeneity from Human Urine Protects Cells from Tumor Necrosis Factor Toxicity," *J. Biol. Chem.,* 264(20):11974–11980 (1989).

Engelmann et al., "Antibodies to a Soluble Form of a Tumor Necrosis Factor (TNF) Receptor Have TNF–Like Activity", *Journal of Biological Chemistry,* 265(24):14497–14504 (1990).

Engelmann et al., "Two Tumor Necrosis Factor–Binding Proteins Purified From Human Urine," *J. Biol. Chem.,* 265(3):1531–1536 (1990).

Feldman et al., "Receptor Activation by Antigens, Cytokines, Hormones, and Growth Factors" *Annals of The New York Academy of Sciences,* 766:272–278 (1995).

Fisher et al., "Treatment of Septic Shock with the Tumor Necrosis Factor Recptor:Fc Fusion Protein," *The New England Journal of Medicine,* 334(26): 1697–1702 (1996).

Gatanaga et al., "Purification and Characterization of an Inhibitor (Soluble Tumor Necrosis Factor Receptor) for Tumor Necrosis Factor and Lymphotoxin Obtained from the Serum Ultrafiltrates of Human Cancer Patients," *Proc. National Academy of Science* USA 87:8781–8784 (1990).

Goodwin et al., "Molecular Cloning and Expression of the Type 1 and Type 2 Murine Receptors for Tumor Necrosis Factor," *Molecular and Cell Biology* 11(6):3020–3026 (1991).

Gray et al., "Cloning of Human Tumor Necrosis Factor (TNF) Receptor cDNA and Expression of Recombinant soluble TNF–Binding Protein," *Proc. Natl. Acad. Sci. USA,* 87(19):7380–7384 (1990).

Hale et al., Cytokines and Their Receptors: From Clonal to Clinical Investigation—"Demonstration of In Vitro and In Vivo Efficacy of Two Biologically Active Human Soluble TNF Receptors Expressed in *E. Coli," J. Cell. Biochem.,* Suppl. 15F:113 (1991).

Harris, Milton, "Laboratory Synthesis of Polyethylene Glycol Derivatives", *Rev. Macromol. Chem. Phys.,* 25(3):325–373 (1985).

Harris et al., "Synthesis and Characterization of Poly(ethylene Glycol) Derivatives," *Journal of Polymer Science-:Polymer Chemistry Edition,* 22:341–352 (1984).

Hauser et al., "Cytokine Accumulations in CSF of Multiple Sclerosis Patients: Frequent Detection of Interleukin–1 and Tumor Necrosis Factor but not Interleukin–6," *Neurology* 40:1735–1739 (1990).

Heller et al., "Complementary DNA Cloning of a Receptor for Tumor Necrosis Factor and Demonstration of a Shed Form of the Receptor," *Proc. Natl. Acad. Sci. USA,* 87:6151–6155 (1990).

Himmler et al., "Molecular Cloning & Expression of Human & Rat Tumor Necrosis Factor Receptor Chain (p60) and Its Soluble Derivative, Tumor Necrosis Factor–Binding Protein," *DNA and Cell Biology,* 9(10):705–715 (1990).

Hofman et al., "Tumor Necrosis Factor Identified in Multiple Sclerosis Brain," *J. Exp. Med.* 170:607–612 (1989).

Hohmann et al., "Two Different Cell Types Have Different Major Receptors for Human Necrosis Factor (TNF α)," *Journal of Biol. Chem.* 264: 14927–14934 (1989).

Katz, *Am. J. Med.*, "Modern Management of Rheumatoid Arthritis", *The American Journal of Medicine,* 79 (suppl 4C):24–31 (1985).

Kavanaugh et al., "Anti–TNG–Alpha Monoclonal Antibody (MAB) Treatment of Rheumatoid Arthritis (RA) Patients with Active Disease on Methotrexate (MTX); Results of a Double–Blind, Placebo Controlled Multicenter Trial", *Arthritis & Rheumatism,* 39(9):S123 (1996).

Kohno et al., "A Second Tumor Necrosis Factor Receptor Gene Product Can Shed a Naturally Occurring Tumor Necrosis Factor Inhibitor," *Proc. Natl. Acad. Sci. USA,* 87:8331–8335 (1990).

Krane and Simon, "Rheumatoid Arthritis: Clinical Features and Pathogenetic Mechanisms", *Advances in Rheumatology,* Synderman (ed.), 70(2):263–284 (1986).

Kull et al., "Cellular Receptor for ^{125}I–Labeled Tumor Necrosis Factor: Specific Binding, Affinity Labeling, and Relationship to Sensitivity," *Proc. Natl. Acad. Sci. USA,* 82:5756–5760 (1985).

Lantz et al., "Characterization In Vitro of a Human Necrosis Factor–Binding Protein," *J. Clin. Invest.,* 86:1396–1402 (1990).

Le et al., "Tumor Necrosis Factor and Interleukin 1: Cytokines with Multiple Overlapping Biological Activities," *Lab Investigation* 56(3):234–248 (1987).

Lehmann et al., "Demonstration of Membrane Receptors for Human Natural and Recombinant ^{125}I–Labeled Tumor Necrosis Factor on HeLa Cell Clones and Their Role in Tumor Cell Sensitivity," *Eur. J. Biochem.* 158:1–5 (1986).

Lindvall et al., "Modulation of the Constitutive Gene Expression of the 55 KD Tumor Necrosis Factor Receptor in Hematopoietic Cells," *Biochem. & Biophys. Res. Comm.* 172(2)557–563 (1990).

Loetscher et al., "Molecular Cloning and Expression of the Human 55kd TNF Necrosis Factor Receptor," *Cell,* 61:351–359 (1990).

Loetscher et al., "Recombinant 55–kDa Tumor Necrosis Factor (TNF) Receptor," *J. Biol. Chem.* 266(27):18324–18329 (1991).

Marsters S. A. et al.: "Identification of Cystein–Rich Domains of the Type 1 Tumor Necrosis Factor Receptor Involved in Ligand Binding", The Journal of Biological Chemistry, 267(9): 5747–5750 (1992).

Moreland et al., "Treatment of Rheumatoid Arthritis with a Recombinant Human Tumor Necrosis Factor Receptor (p75)–Fc Fusion Protein", *New England Journal of Medicine,* 337:141–147 (1997).

Nophar et al., "Soluble forms of tumor necrosis factor receptors (TNF–Rs). The cDNA for the type I TNF–R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor," *The EMBO J.,* 9(10):3269–3278 (1990).

Novick et al., "Soluble Cytokine Receptors are Present in Normal Human Urine," *J. Exp. Med.* 170:1409–1414 (1989).

Novick et al., "Soluble Cytokine Receptors are Present in Normal Human Urine," *The Physiological and Pathological Effects of Cytokines,* pp. 413–421 (1990).

Novick et al., "Purification of Soluble Cytokine Receptors from Normal Human Urine by Ligand–Affinity and Immunoaffinity Chromatography," *J. Chromatog.* 510:331–337 (1990).

Oliff et al., "Tumors Secreting Human TNF/Cachectin Induce Cachexia In Mice", *Cell,* 50:555–563; (1987).

Olsson et al., "Isolation and Characterization of a Tumor Necrosis Factor Binding Protein from Urine," *Eur. J. Haematology,* 42(3):270–275 (1989).

Opal et al., "Potential Hazards of Combination Immunotherapy in the Treatment of Experimental Septic Shock", *The Journal of Infectious Diseases,* 173:1415–1421 (1996).

Paleolog et al., "Deactivation Of Vascular Endothelium By Monoclonal Anti–Tumor Necrosis Factor α Antibody In Rheumatoid Arthritis," *Arthritis & Rheumatism,* 39:1082–1091 (1996).

Peetre et al., "A Tumor Necrosis Factor Binding Protein is Present in Human Biological Fluids," *Eur. J. Haematology,* 41:414–419 (1988).

Pennica et al., "Biochemical Properties of the 75–kDa Tumor Necrosis Factor Receptor", Journal of Biological Chemistry, 267(29):21172–21178 (1992).

Peppel et al., "A Tumor Necrosis Factor (TNF) Receptor–IgG Heavy Chain Chimeric Protein as a Bivalent Antagonist of TNF Activity," *J. Exp. Med.,* 174:1483–1489 (1991).

Rankin et al., "The Therapeutic Effects of an Engineered Human Anti–Tumour Necrosis Factor Alpha Antibody (CDP571) in Rheumatoid Arthritis", *British Journal of Rheumatology,* 34:334–342, (1995).

Rhein, Reginald., "Another Sepsis Drug Down—Immunex' TNF Receptor," Biotechnology *Newswatch,* pp. 1–3 (Monday, Oct. 4, 1983).

Schall et al., "Molecular Cloning and Expression of a Receptor for Human Tumor Necrosis Factor," *Cell,* 61:361–370 (1990).

Scheurich et al., "Quantification and Characterization of a Tumor Necrosis Factor α (TNF–α) Inhibitor: Evidence of Immunological Cross–Reactivity with the TNF Receptor," *Proc. Natl. Acad. Sci. USA,* 87:5188–5192 (1990).

Seckinger et al., "Purification and Biologic Characterization of a Specific Tumor Necrosis Factor α Inhibitor," *J. Biol. Chem.,* 264(20):11966–11973 (1989).

Seckinger et al., "A Urine Inhibitor of Interleukin 1 Activity That Blocks Ligand Binding." *J. Immunol.,* 139(5):1546–1549 (1987).

Seely et al., "Manufacturing of Recombinant Tumor Necrosis Factor Binding Protein 'Dumbbell' Using a 20K PEG BIS–Vinylsulfone Linker," 209th Am. Chem. Soc. National Meeting, Anaheim, Cal., Apr. 2–6, 1995, BIOT 68.

Seitz et al., "Methotrexate in Rheumatoid Arthritis: Stimulation of Cytokine Inhibitor and Inhibition of Chemokine Production by Peripheral Blood Mononuclear Cells", *British Journal of Rheumatology,* 34(7):602–609 (1995).

Seitz et al., "Interleukin 1 (IL–1) Receptor Antagonist, Soluble Tumor Necrosis Factor Receptors, IL–1 beta, and IL–8–markers of Remission in Rheumatoid Arthritis During Treatment with Methotrexate", *Journal of Rheumatology,* 23(9):1512–1516 (1996).

Selby et al. "Endogenous Tumor Necrosis Factor in Cancer Patients", *Lancet,* 1(8583):483 (1988).

Silberberg, "Diseases of Joints", Anderson's Pathology, Kissane (ed.), II:1828–1836 (1985).

Smith, Craig, "cDNA Expression: Cloning of the Receptor for Human Tumor Necrosis Factor." Presentation Programme, 29th Midwinter Conference of Immunologists (Jan. 27–30, 1990).

Smith, Craig, "cDNA Expression: Cloning of the Receptor for Human Tumor Necrosis Factor." Presentation at the 29th Midwinter Conference of Immunologists (Jan. 27–30, 1990).

Smith et al., "A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins," *Science,* 248:1019–1023 (1990).

Smith et al., "A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins," Genbank database excerpt released after publication (May 1990).

Smith et al., "Species Specificity of Human and Murine Tumor Necrosis Factor," *J. Biol. Chem.,* 261(32):14871–14874 (1986).

Spinas et al., "Induction of Plasma Inhibitors of Interleukin 1 and TNF–Alpha Activity by Endotoxin Administration to Normal Humans," *Am. J. Physiol.* 259:R993–R997 (1990).

Starnes, Jr. et al., "Tumor Necrosis Factor and the Acute Metabolic Response to Tissue Injury in Man" 82:1321–1325 (1988).

Stauber et al., "Human Tumor Necrosis Factor–α Receptor," *J. Biol. Chem.,* 263(35):19098–19104 (1988).

Stauber et al., "Characterization and Affinity Cross–Linking of Receptors for Human Recombinant Lymphotoxin (Tumor Necrosis Factor–β) on a Human Histiocytic Lymphoma Cell Line U–937," *J. Biol. Chem.* 264(6):3573–3576 (1989).

Tak et al., "Decrease in Cellularity and Expression of Adhesion Molecules by Anti–Tumor Necrosis Factor α Monoclonal Antibody Treatment in Patients with Rheumatoid Arthritis," *Arthritis and Rheumatism,* 39:1077–1081 (1996).

Tracey et al., "Anti–Cachectin/TNF Monoclonal Antibodies Prevent Septic Shock During Lethal Bacteraemia," *Nature* 330:662–664 (1987).

Tracey et al., "Metabolic Effects of Cachectin/Tumor Necrosis Factor Are Modified by Site of Production," *J. Clin. Invest.* 86:2014–2024 (1990).

Tracey et al., "Physiological responses to cachectin," Tumor necrosis factor and related cytotoxins. Wiley, Chichester (Ciba Foundation Symposium 131), pp. 88–108 (1987).

Tsujimoto et al., "Characterization and Affinity Crosslinking of Receptors for Tumor Necrosis Factor on Human Cells," *Archives of Biochem. & Biophys.* 249(2):563–568 (1986).

Van Zee et al., "Tumor Necrosis Factor Soluble Receptors Circulate During Experimental and Clinical Inflammation and Can Protect Against Excessive Tumor Necrosis Factor α In Vitro and In Vivo", *Proc. Natl. Acad. Sci. USA,* 89:4845–4849 (1992).

Vilcek et al., "Tumor Necrosis Factor: Receptor Binding and Mitogenic Action in Fibroblasts", *Journal of Cellular Physiology Supplement* 5:57–61 (1987).

Vitt et al., "Biological and Structural Characterization of the Tumor Necrosis Factor Receptor on Multiple Cell Types: Relationship to Function", Fed. Proc. 78th Annual Meeting of the American Society of Biological Chemists 46 (6):2117 (Abstract 1118) (1987).

Waage et al., "Association Between Tumour Necrosis Factor in Serum and Fatal Outcome in Patients with Meningococcal Disease", *Lancet,* 1(8529):355 (1987).

Wallach et al., "Cell Surface and Soluble TNF Receptors", Tumor Necrosis Factor: Structure–Function Relationship and Clinical Application, Osawa T, Bonavida B (eds), Karger, Basel. 47–57 (1992).

Wallach et al., "Mechanisms Which Take Part in Regulation of the Response to Tumor Necrosis Factor," *Lymphokine Research* 8(3):359–363 (1989).

Wallach, David, "Preparations of Lymphotoxin Induce Resistance to Their Own Cytotoxic Effect," *J. Immunol.* 132(5):2464–2469 (1984).

Wallach et al., "Regulation of the Response to Tumor Necrosis Factor," Tumor Necrosis Factor/Cachectin and Related Cytokines Int. Conf., Heidelberg 1987, Tumor Necrosis Factor and Related Cytotoxins, Bonavida, Gifford, Kirchner, Old (eds), Karger, Basel. pp. 134–147 (1988).

Yoshie et al., "Binding and Crosslinking of ^{125}I–Labeled Recombinant Human Tumor Necrosis Factor to Cell Surface Receptors," *J. Biochem.* 100:531–541 (1986).

* cited by examiner

FIG. 1

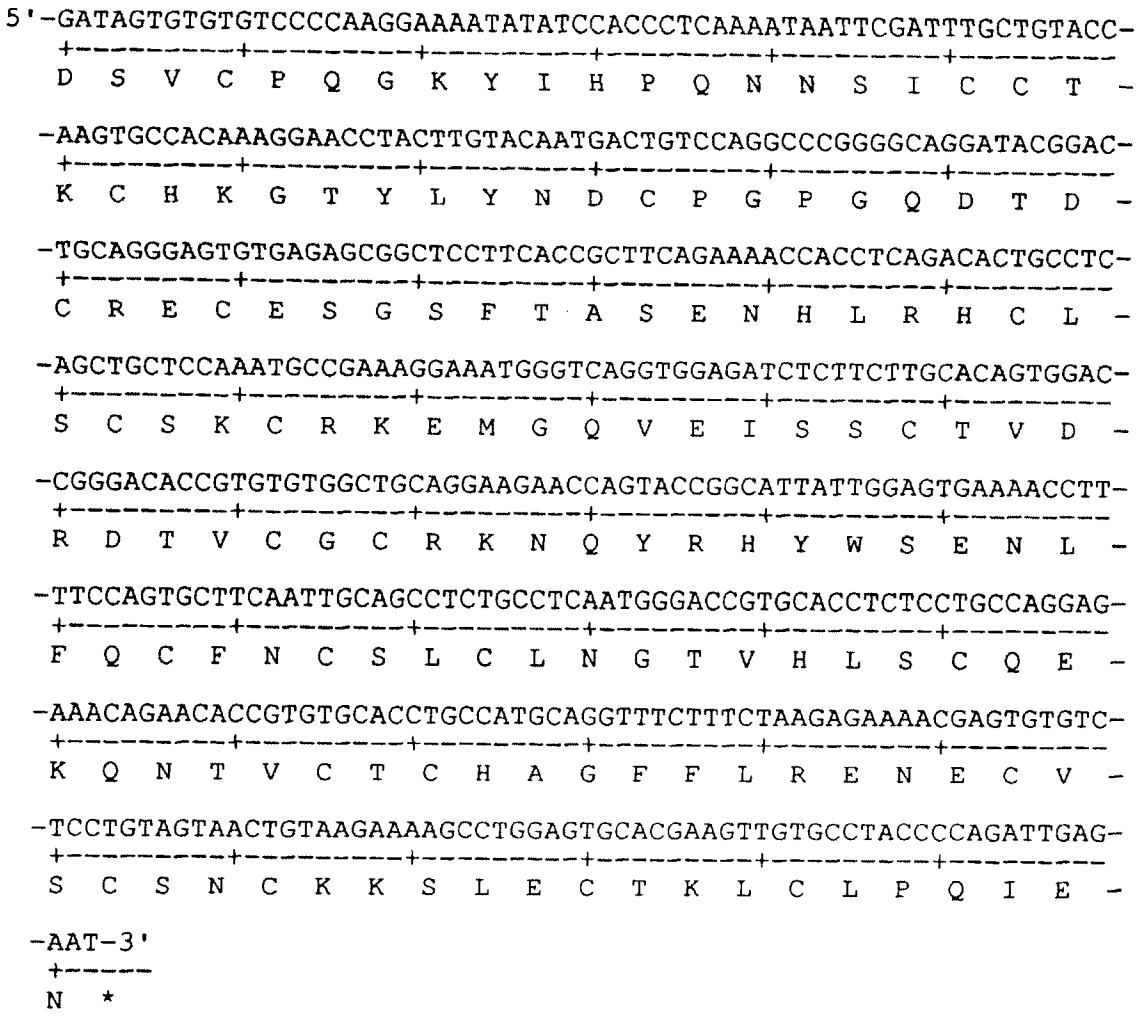

5'-GATAGTGTGTGTCCCCAAGGAAAATATATCCACCCTCAAAATAATTCGATTTGCTGTACC-
D S V C P Q G K Y I H P Q N N S I C C T -
-AAGTGCCACAAAGGAACCTACTTGTACAATGACTGTCCAGGCCCGGGGCAGGATACGGAC-
K C H K G T Y L Y N D C P G P G Q D T D -
-TGCAGGGAGTGTGAGAGCGGCTCCTTCACCGCTTCAGAAAACCACCTCAGACACTGCCTC-
C R E C E S G S F T A S E N H L R H C L -
-AGCTGCTCCAAATGCCGAAAGGAAATGGGTCAGGTGGAGATCTCTTCTTGCACAGTGGAC-
S C S K C R K E M G Q V E I S S C T V D -
-CGGGACACCGTGTGTGGCTGCAGGAAGAACCAGTACCGGCATTATTGGAGTGAAAACCTT-
R D T V C G C R K N Q Y R H Y W S E N L -
-TTCCAGTGCTTCAATTGCAGCCTCTGCCTCAATGGGACCGTGCACCTCTCCTGCCAGGAG-
F Q C F N C S L C L N G T V H L S C Q E -
-AAACAGAACACCGTGTGCACCTGCCATGCAGGTTTCTTTCTAAGAGAAAACGAGTGTGTC-
K Q N T V C T C H A G F F L R E N E C V -
-TCCTGTAGTAACTGTAAGAAAAGCCTGGAGTGCACGAAGTTGTGCCTACCCCAGATTGAG-
S C S N C K K S L E C T K L C L P Q I E -
-AAT-3'
N *

FIG. 2

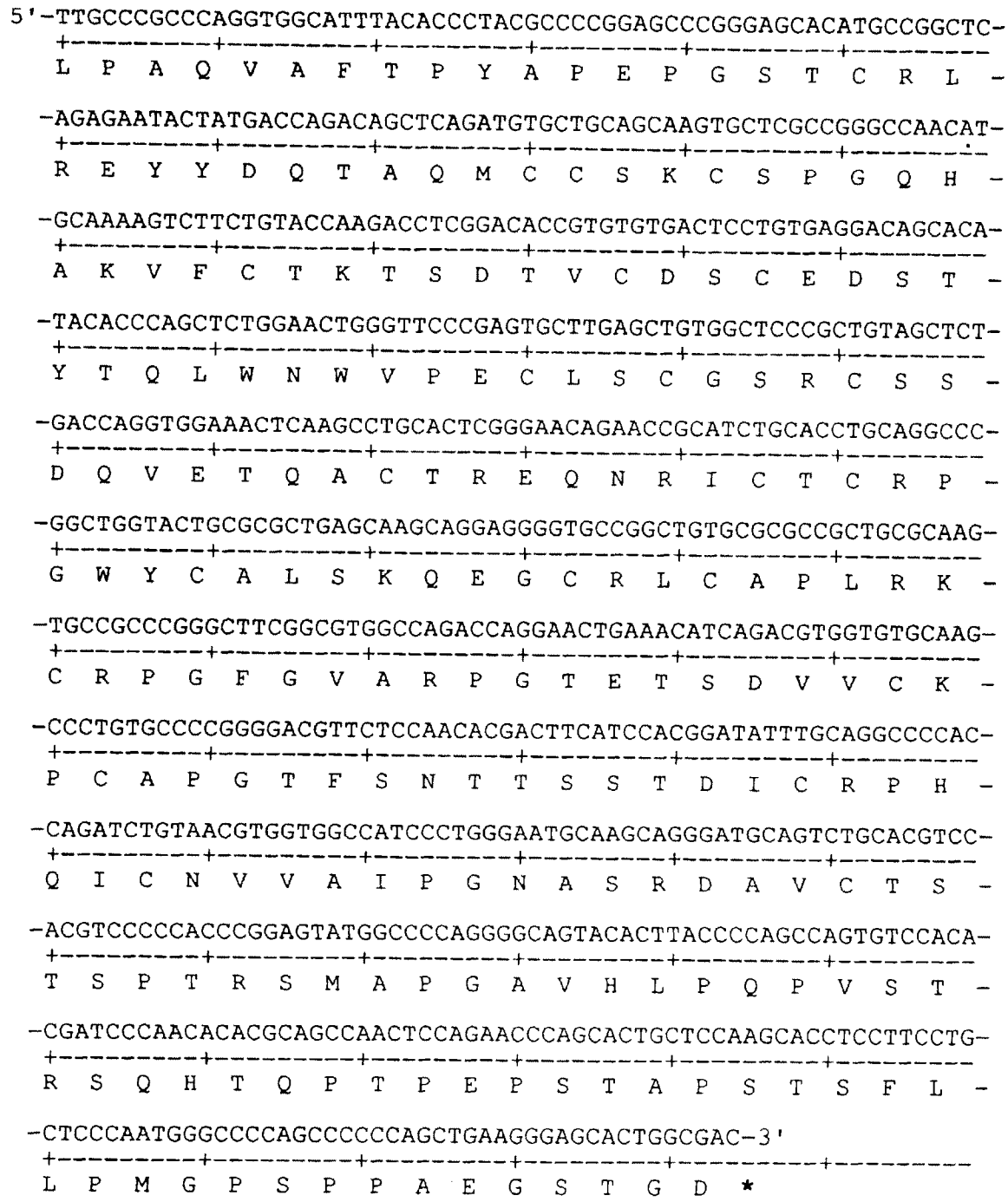

5'-TTGCCCGCCCAGGTGGCATTTACACCCTACGCCCCGGAGCCCGGGAGCACATGCCGGCTC-
L P A Q V A F T P Y A P E P G S T C R L -
-AGAGAATACTATGACCAGACAGCTCAGATGTGCTGCAGCAAGTGCTCGCCGGGCCAACAT-
R E Y Y D Q T A Q M C C S K C S P G Q H -
-GCAAAAGTCTTCTGTACCAAGACCTCGGACACCGTGTGTGACTCCTGTGAGGACAGCACA-
A K V F C T K T S D T V C D S C E D S T -
-TACACCCAGCTCTGGAACTGGGTTCCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCT-
Y T Q L W N W V P E C L S C G S R C S S -
-GACCAGGTGGAAACTCAAGCCTGCACTCGGGAACAGAACCGCATCTGCACCTGCAGGCCC-
D Q V E T Q A C T R E Q N R I C T C R P -
-GGCTGGTACTGCGCGCTGAGCAAGCAGGAGGGGTGCCGGCTGTGCGCGCCGCTGCGCAAG-
G W Y C A L S K Q E G C R L C A P L R K -
-TGCCGCCCGGGCTTCGGCGTGGCCAGACCAGGAACTGAAACATCAGACGTGGTGTGCAAG-
C R P G F G V A R P G T E T S D V V C K -
-CCCTGTGCCCCGGGGACGTTCTCCAACACGACTTCATCCACGGATATTTGCAGGCCCCAC-
P C A P G T F S N T T S S T D I C R P H -
-CAGATCTGTAACGTGGTGGCCATCCCTGGGAATGCAAGCAGGGATGCAGTCTGCACGTCC-
Q I C N V V A I P G N A S R D A V C T S -
-ACGTCCCCCACCCGGAGTATGGCCCCAGGGGCAGTACACTTACCCCAGCCAGTGTCCACA-
T S P T R S M A P G A V H L P Q P V S T -
-CGATCCCAACACACGCAGCCAACTCCAGAACCCAGCACTGCTCCAAGCACCTCCTTCCTG-
R S Q H T Q P T P E P S T A P S T S F L -
-CTCCCAATGGGCCCCAGCCCCCCAGCTGAAGGGAGCACTGGCGAC-3'
L P M G P S P P A E G S T G D *

FIG. 3

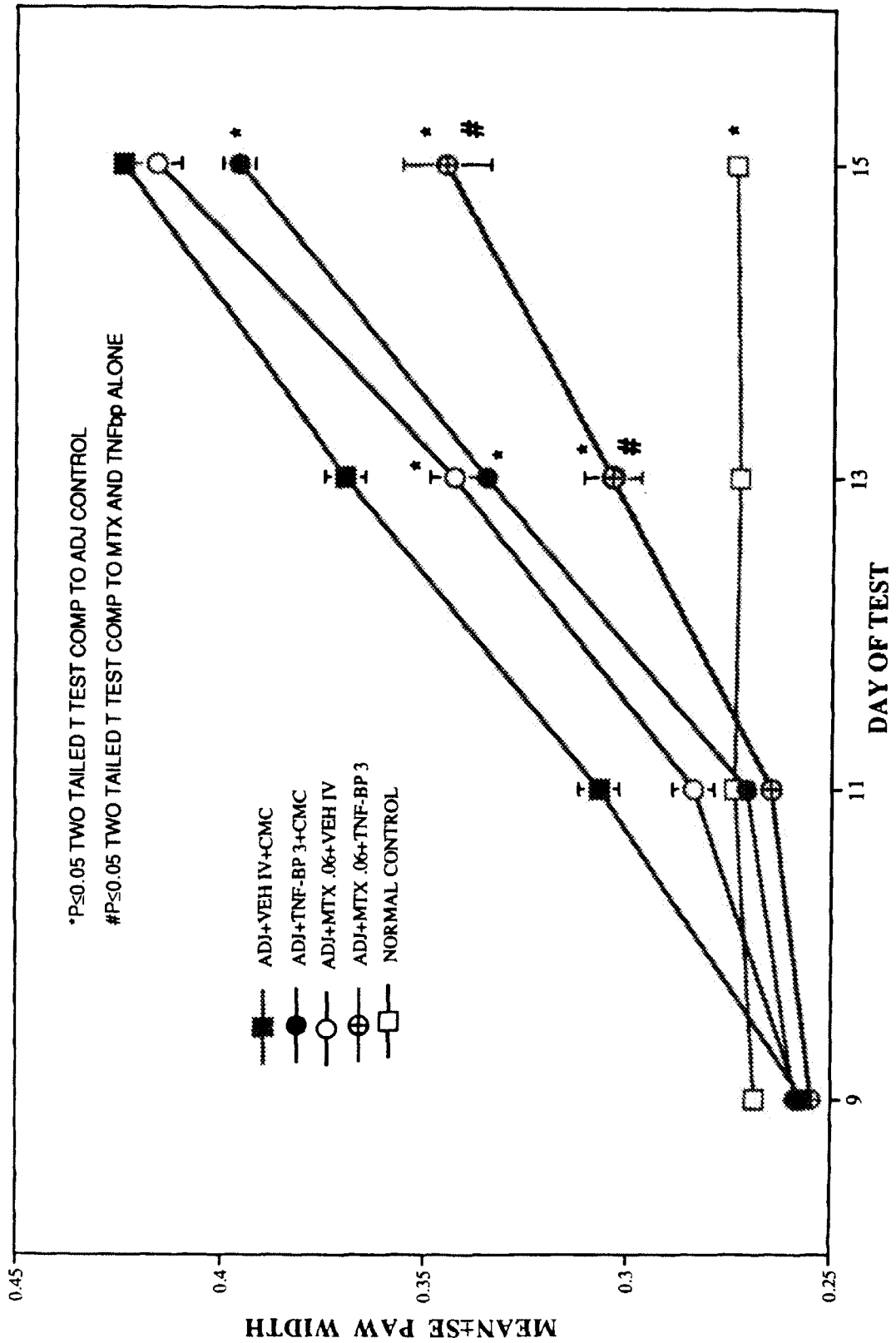

*P≤0.05 TWO TAILED T TEST COMP TO ADJ CONTROL
#P≤0.05 TWO TAILED T TEST COMP TO MTX AND TNFbp ALONE
ADJ+VEH IV+CMC
ADJ+TNF-BP 3+CMC
ADJ+MTX .06+VEH IV
ADJ+MTX .06+TNF-BP 3
NORMAL CONTROL
MEAN±SE PAW WIDTH
0.45
0.4
0.35
0.3
0.25
9
11
13
15
DAY OF TEST

FIG. 4

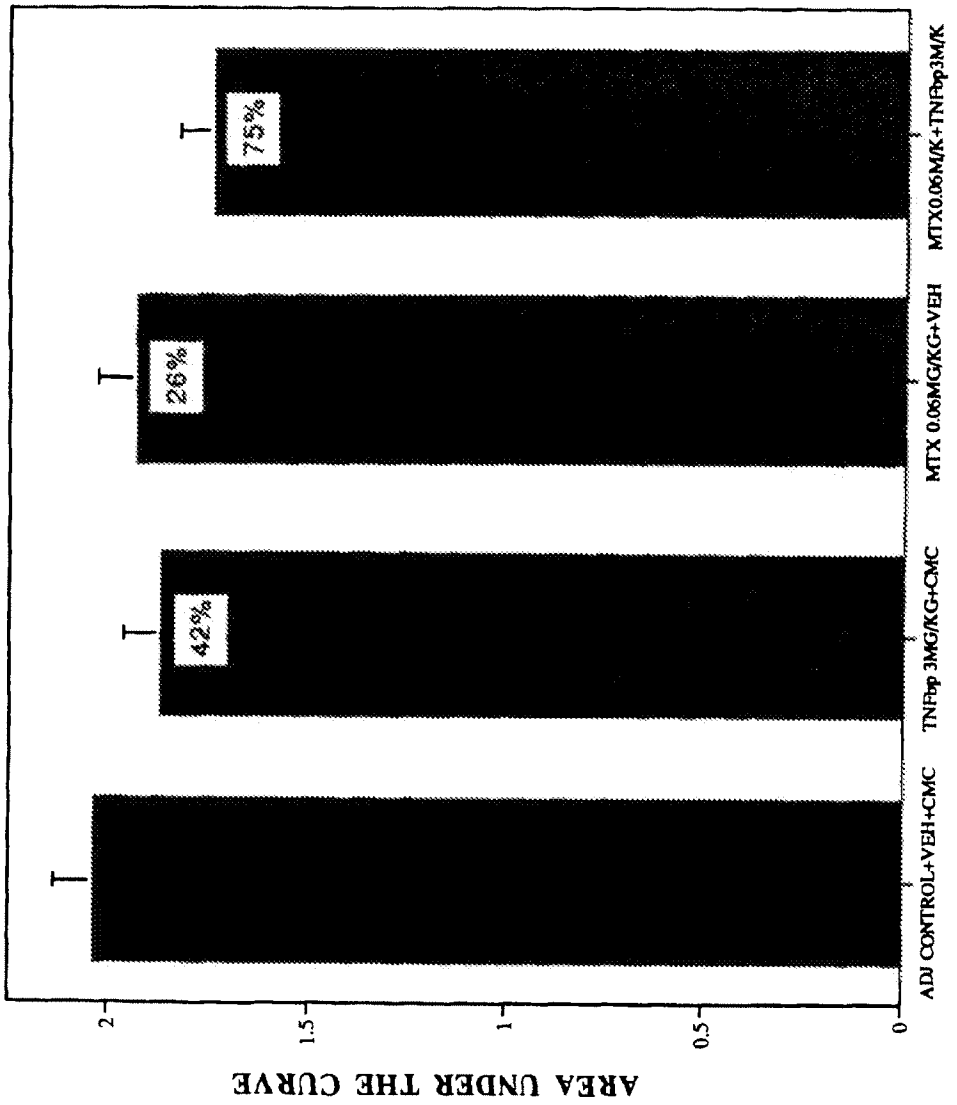

AREA UNDER THE CURVE
2
1.5
1
0.5
0
42%
26%
75%
ADJ CONTROL+VEH+CMC
TNFbp 3MG/KG+CMC
MTX 0.06MG/KG+VEH
MTX0.06M/K+TNFbp3M/K
TREATMENT GROUP

FIG. 5

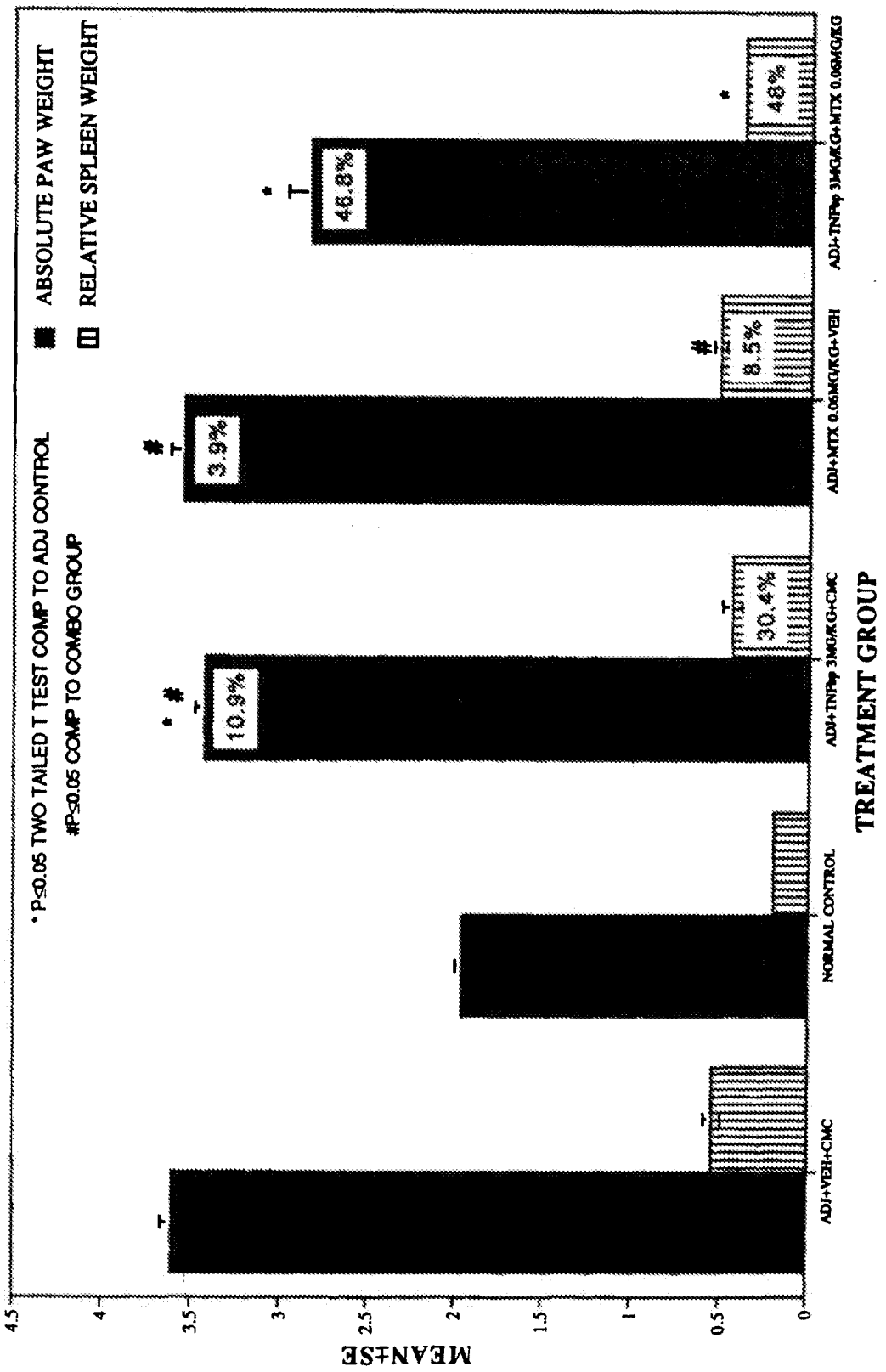

* P≤0.05 TWO TAILED T TEST COMP TO ADJ CONTROL
#P≤0.05 COMP TO COMBO GROUP
ABSOLUTE PAW WEIGHT
RELATIVE SPLEEN WEIGHT
MEAN±SE
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
10.9%
30.4%
3.9%
8.5%
46.8%
48%
ADJ+VEH+CMC
NORMAL CONTROL
ADJ+TNFhp 3MG/KG+CMC
ADJ+MTX 0.06MG/KG+VEH
ADJ+TNFhp 3MG/KG+MTX 0.06MG/KG
TREATMENT GROUP

FIG. 6

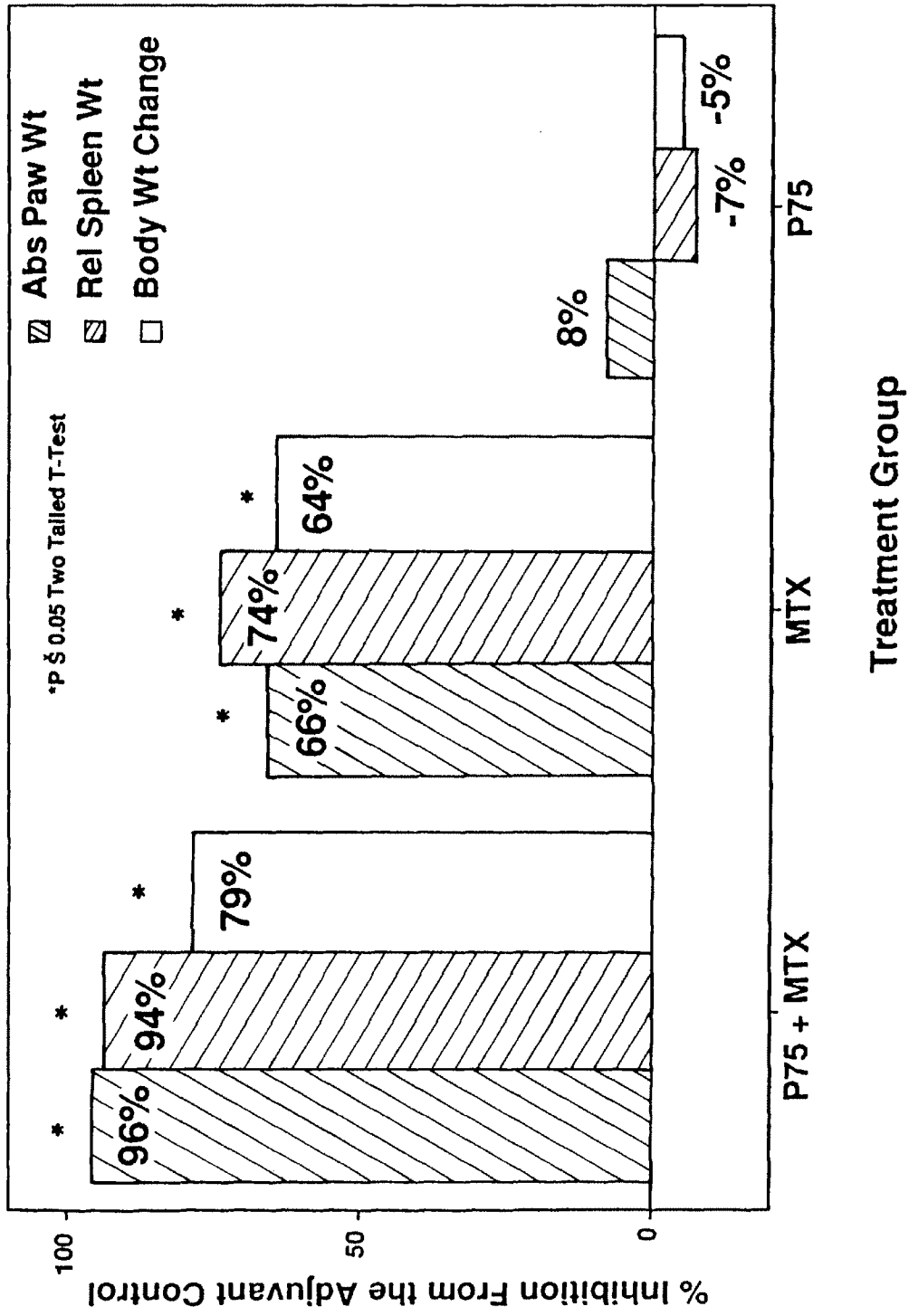

Abs Paw Wt
Rel Spleen Wt
Body Wt Change
*P ≤ 0.05 Two Tailed T-Test
% Inhibition From the Adjuvant Control
100
50
0
96%
94%
79%
66%
74%
64%
8%
-7%
-5%
*
P75 + MTX
MTX
P75
Treatment Group

FIG. 7

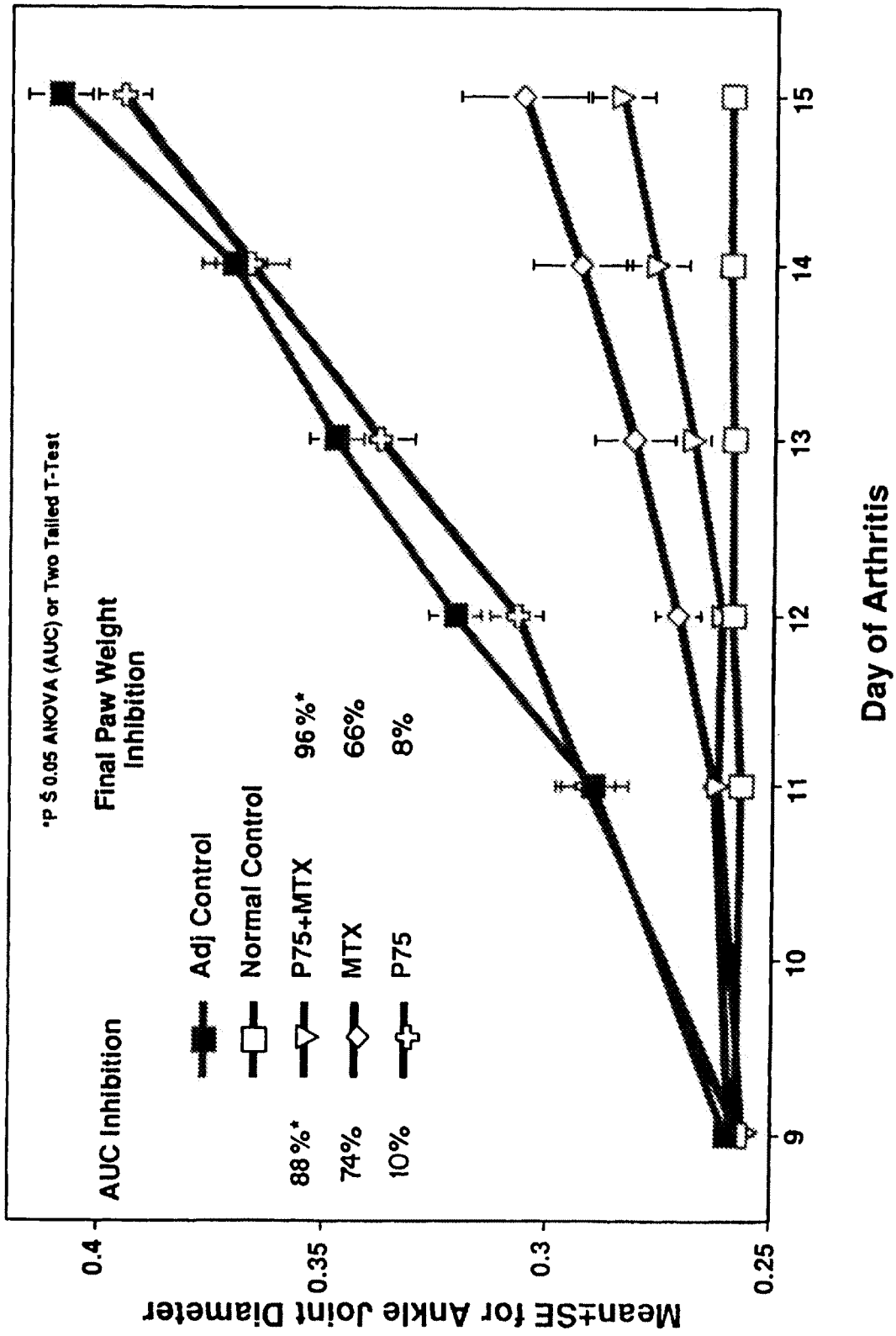
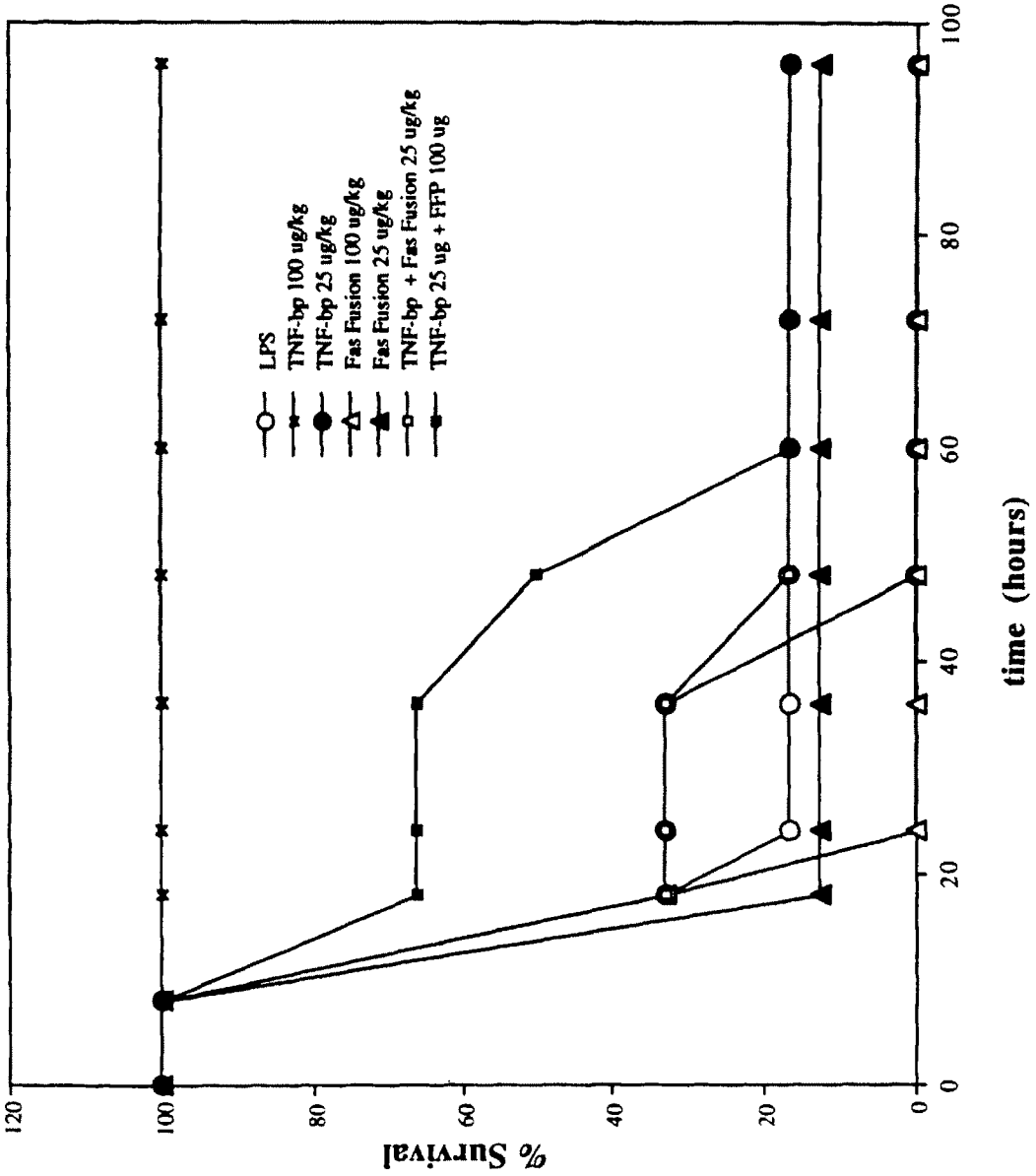

*P ≤ 0.05 ANOVA (AUC) or Two Tailed T-Test
Final Paw Weight Inhibition
AUC Inhibition
Adj Control
Normal Control
P75+MTX
MTX
P75
88%*
74%
10%
96%*
66%
8%
Mean±SE for Ankle Joint Diameter
0.4
0.35
0.3
0.25
9
10
11
12
13
14
15
Day of Arthritis

FIG. 8


% Survival
time (hours)
LPS
TNF-bp 100 ug/kg
TNF-bp 25 ug/kg
Fas Fusion 100 ug/kg
Fas Fusion 25 ug/kg
TNF-bp + Fas Fusion 25 ug/kg
TNF-bp 25 ug + FFP 100 ug
120
100
80
60
40
20
0
0
20
40
60
80
100

COMBINATION THERAPY USING A TNF BINDING PROTEIN FOR TREATING TNF-MEDIATED DISEASES

This application is a continuation of International Application No. PCT/US97/22733, filed Dec. 8, 1997, which claims the benefit of U.S. Provisional Application No. 60/032,587, filed Dec. 6, 1996, U.S. Provisional Application No. 60/036,355, filed Jan. 23, 1997, U.S. Provisional Application No. 60/039,315, filed Feb. 7, 1997 and U.S. Provisional Application No. 60/052,023, filed Jul. 9, 1997, all of which are hereby incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to the field of TNF-mediated diseases. More specifically, the present invention relates to combination therapy for the purpose of preventing or treating TNF-mediated diseases.

BACKGROUND OF THE INVENTION

Inflammation is the body's defense reaction to injuries such as those caused by mechanical damage, infection or antigenic stimulation. An inflammatory reaction may be expressed pathologically when inflammation is induced by an inappropriate stimulus such as an autoantigen, is expressed in an exaggerated manner or persists well after the removal of the injurious agents. Such inflammatory reaction may include the production of certain cytokines.

While the etiology of inflammation is poorly understood, considerable information has recently been gained regarding the molecular aspects of inflammation. This research has led to identification of certain cytokines which are believed to figure prominently in the mediation of inflammation. Cytokines are extracellular proteins that modify the behavior of cells, particularly those cells that are in the immediate area of cytokine synthesis and release. Tumor necrosis factors (TNFs) are a class of cytokines produced by numerous cell types, including monocytes and macrophages.

At least two TNFs have been previously described, specifically TNF alpha (TNF-α) and TNF beta (TNF-β or lymphotoxin), and each is active as a trimeric molecule and is believed to initiate cellular signaling by crosslinking receptors (Engelmann et al. (1990), *J. Biol. Chem.,* 265:14497–14504).

Several lines of evidence implicate TNF-α and TNF-β as major inflammatory cytokines. These known TNFs have important physiological effects on a number of different target cells which are involved in inflammatory responses to a variety of stimuli such as infection and injury. The proteins cause both fibroblasts and synovial cells to secrete latent collagenase and prostaglandin E_2 and cause osteocyte cells to stimulate bone resorption. These proteins increase the surface adhesive properties of endothelial cells for neutrophils. They also cause endothelial cells to secrete coagulant activity and reduce their ability to lyse clots. In addition they redirect the activity of adipocytes away from the storage of lipids by inhibiting expression of the enzyme lipoprotein lipase. TNFs also cause hepatocytes to synthesize a class of proteins known as "acute phase reactants," which act on the hypothalamus as pyrogens (Selby et al. (1988), *Lancet,* 1 (8583):483; Starnes, Jr. et al. (1988), *J. Clin. Invest.,* 82:1321; Oliff et al. (1987), *Cell,* 50:555; and Waage et al. (1987), *Lancet,* 1 (8529):355).

A disease or medical condition is considered to be a "TNF-mediated disease" if the spontaneous or experimental disease is associated with elevated levels of TNF in bodily fluids or in tissues adjacent to the focus of the disease or indication within the body. TNF-mediated diseases may also be recognized by the following two conditions: (1) pathological findings associated with a disease can be mimicked experimentally in animals by the administration of TNF and (2) the pathology induced in experimental animal models of the disease can be inhibited or abolished by treatment with agents which inhibit the action of TNF. Many TNF-mediated diseases satisfy two of these three conditions, and others will satisfy all three conditions.

TNF-mediated diseases such as rheumatoid arthritis and psoriatic arthritis are chronic joint diseases that afflict and disable, to varying degrees, millions of people worldwide. Rheumatoid arthritis is a disease of articular joints in which the cartilage and bone are slowly eroded away by a proliferative, invasive connective tissue called pannus, which is derived from the synovial membrane. The disease may involve peri-articular structures such as bursae, tendon sheaths and tendons as well as extra-articular tissues such as the subcutis, cardiovascular system, lungs, spleen, lymph nodes, skeletal muscles, nervous system (central and peripheral) and eyes (Silberberg (1985), *Anderson's Pathology,* Kissane (ed.), II:1828).

It is believed that rheumatoid arthritis results from the presentation of a relevant antigen to an immunogenetically susceptible host. The antigens that could potentially initiate an immune response resulting in rheumatoid arthritis might be endogenous or exogenous. Possible endogenous antigens include collagen, mucopolysaccharides and rheumatoid factors. Exogenous antigens include mycoplasms, mycobacteria, spirochetes and viruses. By-products of the immune reaction inflame the synovium (i.e., prostaglandins and oxygen radicals) and trigger destructive joint changes (i.e., collagenase).

There is a wide spectrum of disease severity, but many patients run a course of intermittent relapses and remissions with an overall pattern of slowly progressive joint destruction and deformity. The clinical manifestations may include symmetrical polyarthritis of peripheral joints with-pain, tenderness, swelling and loss of function of affected joints; morning stiffness; and loss of cartilage, erosion of bone matter and subluxation of joints after persistent inflammation. Extra-articular manifestations include rheumatoid nodules, rheumatoid vasculitis, pleuropulmonary inflammations, scleritis, sicca syndrome, Felty's syndrome (splenomegaly and neutropenia), osteoporosis and weight loss (Katz (1985), *Am. J. Med.,* 79:24 and Krane and Simon (1986), *Advances in Rheumatology,* Synderman (ed.), 70(2) :263–284). The clinical manifestations result in a high degree of morbidity resulting in disturbed daily life of the patient.

Additionally, preclinical results with various predictive animal models of rheumatoid arthritis have suggested that inhibition of TNF-α can have a major impact on disease progression and severity (Dayer et al. (1994), *European Cytokine Network,* 5(6):563–571 and Feldmann et al. (1995), *Annals Of The New York Academy Of Sciences,* 66:272–278). Moreover, recent human clinical trials in rheumatoid arthritis with inhibitors of TNF have shown promising results (Rankin et al. (1995), *British Journal Of Rheumatology,* 3(4):4334–4342; Elliott et al. (1995), *Lancet,* 344:1105–1110; Tak et al. (1996), *Arthritis and Rheumatism,* 39:1077–1081; Paleolog et al. (1996), *Arthritis and Rheumatism,* 39:1082–1091 and Moreland et al. (1997), *New England Journal of Medicine,* 337:141–147.).

It is an object of the present invention to provide therapeutic methods and compositions for the treatment of TNF-

mediated diseases. This and other objects of the present invention will become apparent from the description hereinafter.

SUMMARY OF THE INVENTION

The present invention relates to therapies for preventing and treating TNF-mediated diseases in a patient. The present invention specifically relates to combination therapy using a TNF binding protein for preventing and treating TNF-mediated diseases, including rheumatic diseases, and the systemic inflammation and body weight loss associated therewith. The type of treatment herein referred to is intended for mammals, including humans.

BRIEF DESCRIPTION OF THE FIGURES

Numerous aspects and advantages of the present invention will become apparent upon review of the figures, wherein:

FIG. **1** depicts a nucleic acid sequence (SEQ ID NO:1) encoding Asp^{1}-Thr^{161}, mature recombinant human soluble TNF receptor type I. Also depicted is the amino acid sequence (SEQ ID NO:2) of Asp^{1}-Thr^{161}. The amino terminus of the amino acid sequence may be methionylated or nonmethionylated.

FIG. **2** depicts a nucleic acid sequence (SEQ ID NO:3) encoding Leu^{1}-Thr^{179}, mature recombinant human soluble TNF receptor type II. Also depicted is the amino acid sequence (SEQ ID NO:4) of Leu^{1}-Thr^{179}. The amino terminus of the amino acid sequence may be methionylated or nonmethionylated.

FIG. **3** depicts the effects of c105 sTNFR-I dumbbell alone, methotrexate alone and the combination of c105 sTNFR-I dumbbell and methotrexate on joint diameter in the adjuvant arthritic rats in Example 1.

FIG. **4** depicts the effects of c105 sTNFR-I dumbbell alone, methotrexate alone and the combination of c105 sTNFR-I dumbbell and methotrexate on final paw weights (index of arthritis), splenomegaly (index of systemic inflammation) and body weight change in the adjuvant arthritic rats in Example 1.

FIG. **5** depicts the final analysis (inhibition at termination) of the effects of c105 sTNFR-I dumbbell alone, methotrexate alone and the combination of c105 sTNFR-I dumbbell and methotrexate on joint diameter in the adjuvant arthritic rats in Example 1.

FIG. **6** depicts the effects of sTNFR-II/Fc alone, methotrexate alone and the combination of sTNFR-II/Fc with methotrexate on final paw weights (index of arthritis), splenomegaly (index of systemic inflammation) and body weight change in the adjuvant arthritic rats in Example 2.

FIG. **7** depicts the effects of sTNFR-II/Fc alone, methotrexate alone and the combination of sTNFR-II/Fc with methotrexate on adjuvant arthritic rats in Example 2.

FIG. **8** depicts the effects of c105 sTNFR-I dumbbell alone, fas fusion protein alone and the combination of c105 sTNFR-I dumbbell and fas fusion protein on LPS/D-Galactosamine lethality in rats in Example 3.

DETAILED DESCRIPTION OF THE INVENTION

The compositions and methods of the invention include administering to a patient afflicted with an inflammatory joint disease an effective amount of a TNF binding protein in combination with any of one or more anti-inflammatory drugs or therapies. The preferred patient is human.

TNF binding proteins are disclosed in the art (EP 308 378, EP 422 339, GB 2 218 101, EP 393 438, WO 90/13575, EP 398 327, EP 412 486, WO 91/03553, EP 418 014, JP 127,800/1991, EP 433 900, U.S. Pat. No. 5,136,021, GB 2 246 569, EP 464 533, WO 92/01002, WO 92/13095, WO 92/16221, EP 512 528, EP 526 905, WO 93/07863, EP 568 928, WO 93/21946, WO 93/19777, EP 417 563, WO 95/34326, WO 96/28546, and PCT Application No. PCT/US97/12244 the disclosures of which are hereby incorporated by reference).

For example, EP 393 438 and EP 422 339 teach the amino acid and nucleic acid sequences of a soluble TNF receptor type I (also known as sTNFR-I or 30 kDa TNF inhibitor) and a soluble TNF receptor type II (also known as sTNFR-II or 40 kDa TNF inhibitor), collectively termed "sTNFRs", as well as modified forms thereof (e.g., fragments, functional derivatives and variants). EP 393 438 and EP 422 339 also disclose methods for isolating the genes responsible for coding the inhibitors, cloning the gene in suitable vectors and cell types, and expressing the gene to produce the inhibitors.

sTNFR-I and sTNFR-II are members of the nerve growth factor/TNF receptor superfamily of receptors which includes the nerve growth factor receptor (NGF), the B cell antigen CD40, 4-1BB, the rat T-cell antigen MRC OX40, the fas antigen, and the CD27 and CD30 antigens (Smith et al. (1990), *Science,* 248:1019–1023). The most conserved feature amongst this group of cell surface receptors is the cysteine-rich extracellular ligand binding domain, which can be divided into four repeating motifs of about forty amino acids and which contains 4–6 cysteine residues at positions which are well conserved (Smith et al. (1990), supra).

For purposes of this invention, sTNFRs and modified forms thereof, including polypeptides in which amino acids of sTNFR-I and sTNFR-II have been deleted from ("deletion variants"), inserted into ("addition variants"), or substituted for ("substitution variants") are collectively termed "TNFbp(s)". [Unless otherwise indicated, amino acid numbering for molecules described herein shall correspond to that presented for the mature form of molecule (i.e., minus the signal sequence), as depicted by amino acids Asp^{1}-Thr^{161} of SEQ ID NO:2, with any initial MET in each such sequence being residue number "0".]

It will be appreciated by those skilled in the art that many combinations of deletions, insertions and substitutions (individually or collectively "variant(s)") can be made within the amino acid sequences of the sTNFRs, provided that the resulting molecule is biologically active (e.g., possesses the ability to bind TNF).

An STNFR variant(s) may be rapidly screened to assess its physical properties. It will be appreciated that such variant(s) will demonstrate similar TNF inhibiting properties, but not necessarily all of the same properties and not necessarily to the same degree as the corresponding unmodified STNFR.

There are two principal variables in the construction of amino acid sequence variant(s): the location of the mutation site and the nature of the mutation. In designing variant(s), the location of each mutation site and the nature of each mutation will depend on the biochemical characteristic(s) to be modified. Each mutation site can be modified individually or in series, e.g., by (1) deleting the target amino acid residue, (2) inserting one or more amino acid residues adjacent to the located site or (3) substituting first with conservative amino acid choices and, depending upon the results achieved, then with more radical selections.

Amino acid sequence deletions generally range from about 1 to 30 amino acid residues, preferably from about 1 to 20 amino acid residues, more preferably from about 1 to 10 amino acid residues and most preferably from about 1 to 5 contiguous residues. Amino-terminal, carboxy-terminal and internal intrasequence deletions are contemplated. Deletions within the amino acid sequences of the sTNFRs may be made, for example, in regions of low homology with the sequences of other members of the NGF/TNF receptor family. Deletions within the amino acid sequences of the sTNFRs in areas of substantial homology with the sequences of other members of the NGF/TNF receptor family will be more likely to significantly modify the biological activity. Specifically, the sequence similarity among NGF/TNF receptor family members is particularly high in the region corresponding to the first two disulfide loops of domain 1, the whole of domain 2, and the first disulfide loop of domain 3 (Banner et al. (1993), *Cell,* 73:431–445). The number of total deletions and/or consecutive deletions preferably will be selected so as to preserve the tertiary structure in the affected domain, e.g., cysteine crosslinking.

EP 393 438 teaches a 40 kDa TNF inhibitor Δ51 and a 40 kDa TNF inhibitor Δ53, which are truncated versions of the full-length recombinant 40 kDa TNF inhibitor protein wherein 51 or 53 amino acid residues, respectively, at the carboxyl terminus of the mature protein are removed. Accordingly, a skilled artisan would appreciate that the fourth domain of each of the 30 kDa TNF inhibitor and the 40 kDa inhibitor is not necessary for TNF inhibition. In fact various groups have confirmed this understanding. Domain-deletion derivatives of the 30 kDa and 40 kDa TNF inhibitors have been generated, and those derivatives without the fourth domain retain full TNF binding activity while those derivatives without the first, second or third domain, respectively, do not retain TNF binding activity (Corcoran et al. (1994), *Eur. J. Biochem.,* 223:831–840; Chih-Hsueh et al. (1995), *The Journal of Biological Chemistry,* 270(6): 2874–2878; and Scallon et al. (1995), *Cytokine,* 7(8): 759–770).

PCT Application No. PCT/U.S.97/12244 teaches truncated forms of sTNFR-I and sTNFR-II which do not contain the fourth domain (amino acid residues Thr^{127}-Asn^{161} of sTNFR-I and amino acid residues Pro^{141}-Thr^{179} of sTNFR-II); a portion of the third domain (amino acid residues Asn^{111}-Cys^{126} of sTNFR-I and amino acid residues Pro^{123}-Lys^{140} of sTNFR-II); and, optionally, which do not contain a portion of the first domain (amino acid residues Asp^{1}-Cys^{19} of sTNFR-I and amino acid residues Leu^{1}-Cys^{32} of sTNFR-II). The truncated sTNFRs of the present invention include the proteins represented by the formula R_1-[Cys^{19}-Cys^{103}]-R_2 and R_4-[Cys^{32}-Cys^{115}]-R_5. These proteins are truncated forms of sTNFR-I and sTNFR-II, respectively.

By "R_1-[Cys^{19}-Cys^{103}]-R_2" is meant one or more proteins wherein [Cys^{19}-Cys^{103}] represents residues 19 through 103 of sTNFR-I, the amino acid residue numbering scheme of which is provided in FIG. **1** to facilitate the comparison; wherein R_1 represents a methionylated or nonmethionylated amine group of Cys^{19} or of amino-terminus amino acid residue(s) selected from any one of Cys^{18} to Asp^{1} and wherein R_2 represents a carboxy group of Cys^{103} or of carboxy-terminal amino acid residues selected from any one of Phe^{104} to Leu^{110}.

Exemplary truncated sTNFR-I of the present invention include the following molecules (collectively termed 2.6 D sTNFR-I): NH_2-[Asp^{1}-Cys^{105}]-COOH (also referred to as sTNFR-I 2.6 D/C105); NH_2-[Asp^{1}-Leu^{108}]-COOH (also referred to as sTNFR-I 2.6 D/C106); NH_2-[Asp^{1}-Asn^{105}]-COOH (also referred to as sTNFR-I 2.6 D/N105); NH_2-[Tyr^{9}-Leu^{108}]-COOH (also referred to as sTNFR-I 2.3 D/d8); NH_2-[Cys^{19}-Leu^{108}]-COOH (also referred to as sTNFR-I 2.3 D/d18); and NH_2-[Ser^{16}-Leu^{108}]-COOH (also referred to as sTNFR-I 2.3 D/d15), either methionylated or nonmethionylated, and variants and derivatives thereof.

By "R_3-[Cys^{32}-Cys^{115}]-R_4" is meant one or more proteins wherein [Cys^{32}-Cys^{115}] represents residues Cys^{32} through Cys^{115} of sTNFR-II, the amino acid residue numbering scheme of which is provided in FIG. **2** to facilitate the comparison; wherein R_3 represents a methionylated or nonmethionylated amine group of Cys^{32} or of amino-terminus amino acid residue(s) selected from any one of Cys^{31} to Leu^{1} and wherein R_4 represents a carboxy group of Cys^{115} or of carboxy-terminal amino acid residue(s) selected from any one of Ala^{116} to Arg^{122}.

An amino acid sequence addition may include insertions of an amino- and/or carboxyl-terminal fusion ranging in length from one residue to one hundred or more residues, as well as internal intrasequence insertions of single or multiple amino acid residues. Internal additions may range generally from about 1 to 20 amino acid residues, preferably from about 1 to 10 amino acid residues, more preferably from about 1 to 5 amino acid residues, and most preferably from about 1 to 3 amino acid residues. Additions within the amino acid sequences of the sTNFRs may be made in regions of low homology with the sequences of other members of the NGF/TNF receptor family. Additions within the amino acid sequence of the sTNFRs in areas of substantial homology with the sequences of other members of the NGF/TNF receptor family will be more likely to significantly modify the biological activity. Additions preferably include amino acid sequences derived from the sequences of the NGF/TNF receptor family members.

An amino-terminus addition is contemplated to include the addition of a methionine (for example, as an artifact of the direct expression in bacterial recombinant cell culture). A further example of an amino-terminal addition includes the fusion of a signal sequence to the amino-terminus of mature sTNFRs in order to facilitate the secretion of protein from recombinant host cells. Such signal sequences generally will be obtained from and thus be homologous to the intended host cell species. For prokaryotic host cells that do not recognize and process the native signal sequence of the sTNFRs, the signal sequence may be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase or heat-stable enterotoxin II leader sequences. For expression in yeast cells the signal sequence may be selected, for example, from the group of the yeast invertase, alpha factor or acid phosphatase leader sequences. In mammalian cell expression the native signal sequences (EP 393 438 and EP 422 339) are satisfactory, although other mammalian signal sequences may be suitable, for example sequences derived from other NGF/TNP receptor family members.

An example of an amino- or a carboxy-terminus addition includes chimeric proteins comprising the amino-terminal or carboxy-terminal fusion of a TNFbp(s) with all or part of the constant domain of the heavy or light chain of human immunoglobulin (individually or collectively, ("sTNFR Fc(s)"). Such chimeric polypeptides are preferred wherein the immunoglobulin portion of each comprises all of the domains except the first domain of the constant region of the heavy chain of human immunoglobulin such as IgG (e.g., IgG1 or IgG3), IgA, IgM or IgE. A skilled artisan will appreciate that any amino acid of the immunoglobulin portion can be deleted or substituted with one or more amino

acids, or one or more amino acids can be added as long as the TNF binding protein portion still binds TNF and the immunoglobulin portion shows one or more of its characteristic properties.

Another group of variant(s) is amino acid substitution variant(s) of the amino acid sequence of sTNFRs. These are variant(s) wherein at least one amino acid residue in an sTNFR is removed and a different residue inserted in its place. Substitution variant(s) include allelic variant(s) which are characterized by naturally-occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change. One skilled in the art can use any information known about the binding or active site of the polypeptide in the selection of possible mutation sites.

One method for identifying amino acid residues or regions for mutagenesis of a protein is called "alanine scanning mutagenesis", as described by Cunningham and Wells (1989), *Science,* 244:1081–1085, the disclosure of which is hereby incorporated by reference. In this method, an amino acid residue or group of target residues is identified (e.g., charged residues such as Arg, Asp, His, Lys and Glu) and replaced by a neutral or negatively-charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains/residues demonstrating functional sensitivity to the substitutions are then refined by introducing additional or alternate residues at the sites of substitution. Thus, the site for introducing an amino acid sequence modification is predetermined. To optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted and the variant(s) may be screened for the optimal combination of desired activity and degree of activity.

The sites of greatest interest for substitutional mutagenesis include sites in which particular amino acid residues within an sTNFR are substantially different from other species or or other NGF/TNF receptor family members in terms of side-chain bulk, charge and/or hydrophobicity. Other sites of interest include those in which particular residues of an sTNFR are identical among other species or other NGF/TNF receptor family members, as such positions are generally important for the biological activity of a protein.

Other sites of interest include those in which particular residues are similar or identical with those of such sTNFR-I-like proteins and sTNFR-II-like proteins. Accordingly, the following information has been elucidated concerning sTNFR-I (Banner et al. (1993), supra, and Fu et al. (1995), *Protein Engineering,* 8(12):1233–1241). Residues Tyr^{9}, Thr^{39}, His^{55} in Domain 1, residues Phe^{49}, Ser^{63}, Asp^{82} in Domain 2 and residues Tyr^{92} and Ser^{107} in Domain 3 have been identified as being potentially important for the stabilization of the structure of Domains 1, 2 and 3, respectively. Residues Pro^{12} and His^{55} have been identified as potentially interacting with Ser^{86}-Tyr^{87} on subunit C of TNF-α. Residues Glu^{45}-Phe^{49} have been identified as being in a loop which potentially interacts with residues Leu^{29}-Arg^{32} of TNF-α subunit A. Residues Gly^{48} has been identified as potentially interacting with Asn^{19}-Pro^{20} on subunit A of TNF-α. Residue His^{58}-Leu^{60} have been identified as being in an extended strand conformation and side chain interactions with residues Arg^{31}-Ala^{33} on subunit A of TNF-α have been potentially identified with residue His^{58} of sTNFR-I specifically interacting with residue Arg^{31}. Residues Lys^{64}-Arg^{66} have been identified as being in an extended strand conformation and have been identified as having side chain and main chain interactions with residues Ala^{145}-Glu^{146} and residue Glu^{46} on subunit A of TNF-α. Residue Met^{69} has been identified as potentially interacting with residue Tyr^{115} on subunit A of TNF-α. Residues His^{94}-Phe^{101} have been identified as forming a loop which interacts with residues Thr^{72}-Leu^{75} and Asn^{137} of subunit C of TNF-α, with residue Trp^{96} of sTNFR-I specifically interacting with residues Ser^{71}-Thr^{72} on subunit C of TNF-α, Leu^{100} of STNFR-I being in close proximity with residue Asn^{137} on subunit C of TNF-α and residue Gln^{102} of sTNFR-I specifically interacting with residue Pro^{113} on subunit A of TNF-α.

In addition to the cysteines forming the 3 pairs of disulfide bonds within each of the four domains of the molecule, there are several other conserved residues that contribute to the stabilization of the tertiary fold of each domain.

There are two main classes into which these stabilizing residues fall. The first type contributes to the shielding of the disulfide bond sulfur atoms from solvent. An example of this residues in domain 3 is Tyr^{92}. In domain 4 Phe^{133} helps to shield the Cys^{128}-Cys^{139} disulfide bond. All four domains have either a Tyr or Phe at these same structurally conserved locations. The second class of stabilizing residues form hydrogen bonds within their respective domains. Within domain 3 Asn^{123} and Ser^{107} form a hydrogen bond and Ser^{107} forms an additional hydrogen bond with Thr^{124}. For domain 4 these residues include Asn^{144} and Ser^{141}.

In addition there are hydrogen bonds between domain 3 and 4 that are not seen between other domains. These hydrogens bonds are (1) Asn^{105} main-chain oxygen and Asn^{137} side-chain nitrogen and (2) Ser^{107} side-chain oxygen and Asn^{137} main-chain nitrogen.

A skilled artisan will appreciate that initially the sites should be modified by substitution in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "Preferred Substitutions". If such substitutions result in a change in biological activity, then more substantial changes (Exemplary Substitutions) may be introduced and/or other additions/deletions may be made and the resulting products screened.

TABLE 1

Amino Acid Substitutions

Original Residue	Preferred Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Lys; Arg
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; norleucine
Leu (L)	Ile	norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Leu	Leu; Val; Ile; Ala
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr

TABLE 1-continued

Amino Acid Substitutions		
Original Residue	Preferred Substitutions	Exemplary Substitutions
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; norleucine

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle (1982), *J. Mol. Biol.,* 157:105–131, the disclosure of which is incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the functionally equivalent protein or peptide thereby created is intended for use in immunological embodiments, as in the present case. U.S. Pat. 4,554,101, the disclosure of which is incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e., with a biological property of the protein.

U.S. Pat. 4,554,101 also teaches the identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity. Through the methods disclosed in U.S. Pat. 4,554,101 a skilled artisan would be able to identify epitopes, for example, within the amino acid sequence of an sTNFR. These regions are also referred to as "epitopic core regions". Numerous scientific publications have been devoted to the prediction of secondary structure, and to the identification of epitopes, from analyses of amino acid sequences (Chou and Fasman (1974), *Biochemistry,* 13(2):222–245; Chou and Fasman (1974), *Biochemistry,* 13(2):211–222; Chou and Fasman (1978), *Adv. Enzymol. Relat. Areas Mol. Biol.,* 47:45–148; Chou and Fasman (1978), *Ann. Rev. Biochem.,* 47:251–276 and Chou and Fasman (1979), *Biophys. J.,* 26:367–384, the disclosures of which are incorporated herein by reference). Moreover, computer programs are currently available to assist with predicting antigenic portions and epitopic core regions of proteins. Examples include those programs based upon the Jameson-Wolf analysis (Jameson and Wolf (1988), *Comput. Appl. Biosci.,* 4(1):181–186 and Wolf et al. (1988), *Comput. Appl. Biosci.,* 4(1):187–191, the disclosures of which are incorporated herein by reference); the program PepPlot® (Brutlag et al. (1990), *CABS,* 6:237–245 and Weinberger et al. (1985), *Science,* 228:740–742, the disclosures of which are incorporated herein by reference); and other programs for protein tertiary structure prediction (Fetrow and Bryant (1993), *BIOTECHNOLOGY,* 11:479–483, the disclosure of which is incorporated herein by reference).

In contrast, substantial modifications in the functional and/or chemical characteristics of the sTNFRs may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the relative charge or hydrophobicity of the protein at the target site or (c) the bulk of the side chain. Naturally-occurring residues are divided into groups based on common side chain properties:

1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
2) neutral hydrophilic: Cys, Ser, Thr;
3) acidic: Asp, Glu;
4) basic: Asn, Gln, His, Lys, Arg;
5) aromatic: Trp, Tyr, Phe; and
6) residues that influence chain orientation: Gly, Pro.

Non-conservative substitutions may involve the exchange of a member of one of these groups for another. Such substituted residues may be introduced into regions of the sTNFRs that, for example, are homologous with other NGF/TNF receptor family members or into non-homologous regions of the protein.

A variety of amino acid substitutions or deletions may be made to modify or add N-linked or O-linked glycosylation sites, resulting in a protein with altered glycosylation. The sequence may be modified to add glycosylation sites to or to delete N-linked or O-linked glycosylation sites from the sTNFRs. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro. Proven or predicted asparagine residues of 30 kDa TNF inhibitor exist at positions 14, 105 and 111.

Specific mutations of the sequences of the sTNFRs may involve substitution of a non-native amino acid at the amino-terminus, carboxy-terminus or at any site of the protein that is modified by the addition of an N-linked or O-linked carbohydrate. Such modifications may be of particular utility in the addition of an amino acid (e.g., cysteine), which is advantageous for the linking of a water soluble polymer to form a derivative. For example, WO 92/16221 describes the preparation of sTNFR-I muteins, e.g., wherein an asparagine residue at position 105 of the native human protein is changed to cysteine (c105 sTNFR-I).

In a specific embodiment, a variant polypeptide will preferably be substantially homologous to the amino acid of the sTNFR from which it is derived. The term "substantially homologous" as used herein means a degree of homology that is in excess of 80%, preferably in excess of 90%, more preferably in excess of 95% or most preferably even 99%. The percentage of homology as described herein is calculated as the percentage of amino acid residues found in the smaller of the two sequences which align with identical amino acid residues in the sequence being compared when four gaps in a length of 100 amino acids may be introduced to assist in that alignment, as set forth by Dayhoff (1972), *Atlas of Protein Sequence and Structure,* 5:124, National Biochemical Research Foundation, Washington, D.C., the disclosure of which is hereby incorporated by reference. Also included within the term "substantially homologous" are variant(s) of sTNFRs which may be isolated by virtue of cross-reactivity with antibodies to the amino acid sequences of SEQ ID NO:2 and SEQ ID NO:4 or whose genes may be isolated through hybridization with the DNA of SEQ ID NO:1 and SEQ ID NO:3 or with segments thereof.

Polypeptide Derivatives

Chemically-modified derivatives of the TNFbp(s) in which the protein is linked to a polymer in order to modify properties of the protein (referred herein as "derivatives")

are included within the scope of the present invention. Such derivatives may be prepared by one skilled in the art given the disclosures herein. Conjugates may be prepared using glycosylated, non-glycosylated or de-glycosylated TNFbp (s) and suitable chemical moieties. Typically non-glycosylated proteins and water soluble polymers will be used.

Water soluble polymers are desirable because the protein to which each is attached will not precipitate in an aqueous environment, such as a physiological environment. Preferably, the polymer will be pharmaceutically acceptable for the preparation of a therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically and, if so, the therapeutic profile of the protein (e.g., duration of sustained release; resistance to proteolysis; effects, if any, on dosage; biological activity; ease of handling; degree or lack of antigenicity and other known effects of a water soluble polymer on a therapeutic proteins).

Suitable, clinically acceptable, water soluble polymers include, but are not limited to, polyethylene glycol (PEG), polyethylene glycol propionaldehyde, copolymers of ethylene glycol/propylene glycol, monomethoxy-polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol (PVA), polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, poly(β-amino acids) (either homopolymers or random copolymers), poly(n-vinyl pyrrolidone)polyethylene glycol, polypropylene glycol homopolymers (PPG) and other polyalkylene oxides, polypropylene oxide/ethylene oxide copolymers, polyoxyethylated polyols (POG) (e.g., glycerol) and other polyoxyethylated polyols, polyoxyethylated sorbitol, or polyoxyethylated glucose, colonic acids or other carbohydrate polymers, Ficoll or dextran and mixtures thereof. As used herein, polyethylene glycol is meant to encompass any of the forms that have been used to derivatize other proteins, such as mono-(C1–C10) alkoxy- or aryloxy-polyethylene glycol. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

The water soluble polymers each may be of any molecular weight and may be branched or unbranched. Generally, the higher the molecular weight or the more branches, the higher the polymer:protein ratio. The water soluble polymers each typically have an average molecular weight of between about 2 kDa to about 100 kDa (the term "about" indicating that in preparations of a water soluble polymer, some molecules will weigh more, some less, than the stated molecular weight). The average molecular weight of each water soluble polymer preferably is between about 5 kDa and about 40 kDa, more preferably between about 10 kDa and about 35 kDa and most preferably between about 15 kDa and about 30 kDa.

There are a number of attachment methods available to those skilled in the art, including acylation reactions or alkylation reactions (preferably to generate an amino-terminal chemically modified protein) with a reactive water soluble molecule. See, for example, EP 0 401 384; Malik et al. (1992), *Exp. Hematol.,* 20:1028–1035; Francis (1992), *Focus on Growth Factors,* 3(2):4–10, published by Mediscript, Mountain Court, Friern Barnet Lane, London N20 OLD, UK; EP 0 154 316; EP 0 401 384; WO 92/16221; WO 95/34326; WO 95/13312; WO 96/11953; WO 96/19459 and WO 96/19459 and the other publications cited herein that relate to pegylation, the disclosures of which are hereby incorporated by reference.

A specific embodiment of the present invention is an unbranched monomethoxy-polyethylene glycol aldehyde molecule having an average molecular weight of either about 20 kDa or about 33 kDa (e.g., between 30 kDa and 35 kDa), or a tertiary-butyl polyethylene glycol aldehyde having an average molecular weight of about 33 kDa (e.g., between 30 kDa and 35 kDa) conjugated via reductive alkylation to the TNFbp(s).

The pegylation also may be specifically carried out using water soluble polymers having at least one reactive hydroxy group (e.g. polyethylene glycol). The water soluble polymer can be reacted with an activating group, thereby forming an "activated linker" useful in modifying various proteins. The activated linkers can be monofunctional, bifunctional, or multifunctional.

Activating groups which can be used to link the water soluble polymer to two or more proteins include the following: sulfone, maleimide, sulfhydryl, thiol, triflate, tresylate, azidirine, oxirane and 5-pyridyl. Useful reagents having a reactive sulfone group that can be used in the methods include, without limitation, chlorosulfone, vinylsulfone and divinylsulfone. These PEG derivatives are stable against hydrolysis for extended periods in aqueous environments at pHs of about 11 or less, and can form linkages with molecules to form conjugates which are also hydrolytically stable. Two particularly useful homobifunctional derivatives are PEG-bis-chlorosulfone and PEG-bis-vinylsulfone (WO 95/13312).

WO 97/04003, the disclosure of which is hereby incorporated by reference, teaches methods of making sulfone-activated linkers by obtaining a compound having a reactive hydroxyl group and converting the hydroxyl group to a reactive Michael acceptor to form an activated linker, with the use of tetrahydrofuran (THF) as the solvent for the conversion. The application also teaches a process for purifying the activated linkers which utilizes hydrophobic interaction chromatography to separate the linkers based on size and end-group functionality.

Polyvalent Forms

Polyvalent forms, i.e., molecules comprising more than one active moiety, may be constructed. In one embodiment, the molecule may possess multiple tumor necrosis factor binding sites for the TNF ligand. Additionally, the molecule may possess at least one tumor necrosis factor binding site and, depending upon the desired characteristic of polyvalent form, at least one site of another molecule (e.g., a TNFbp(s), and an interleukin-1 receptor antagonist ("IL-lra") as described below).

In one embodiment, the polyvalent form may be constructed, for example, by chemically coupling at least one TNFbp(s) and another moiety with any clinically accepted linker (e.g., a water-soluble polymer). In principle the linker must not impart new immunogenecity nor, by virtue of the new amino acid residues, alter the hydrophobicity and charge balance of the structure which affects its biodistribution and clearance.

The water soluble polymers can be, based on the monomers listed herein, homopolymers, random or block copolymers, terpolymers straight chain or branched, substituted or unsubstituted. The polymer can be of any length or molecular weight, but these characteristics can affect the biological properties. Polymer average molecular weights particularly useful for decreasing clearance rates in pharmaceutical applications are in the range of 2,000 to 35,000 daltons. In addition, the length of the polymer can be varied to optimize or confer the desired biological activity.

The active moieties may be linked using conventional coupling techniques (see WO 92/16221, WO 95/13312 and

WO 95/34326, the disclosures of which are hereby incorporated by reference). For example, WO 92/16221 and WO 95/34326 describe the preparation of various dimerized sTNFR-I molecules, e.g., dimerized c105 STNFR-I.

Alternatively, a bivalent molecule may consist of two tandem repeats of sTNFRs separated by a polypeptide linker region. The design of the polypeptide linkers is similar in design to the insertion of short loop sequences between domains in the de novo design of proteins (Mutter (1988), *TIBS,* 13:260–265 and Regan and DeGrado (1988), *Science,* 241:976–978, the disclosures of which are hereby incorporated by reference). Several different linker constructs have been assembled and shown to be useful for forming single chain antibodies; the most functional linkers vary in size from 12 to 25 amino acids (amino acids having unreactive side groups, e.g., alanine, serine and glycine) which together constitute a hydrophilic sequence, have a few oppositely charged residues to enhance solubility and are flexible (Whitlow and Filpula (1991), *Methods: A Companion to Methods in Enzymology,* 2:97–105; and Brigido et al. (1993), *J. Immunol.,* 150:469–479, the disclosures of which are hereby incorporated by reference). It has been shown that a linker suitable for single chain antibodies is effective to produce a dimeric form of the human sTNFR-II (Neve et al. (1996), *Cytokine,* 8(5):365–370, the disclosure of which is hereby incorporated by reference).

Additionally, a TNFbp(s) may be chemically coupled to biotin, and the resulting conjugate may then be allowed to bind to avidin, resulting in tetravalent avidin/biotin/TNFbp (s) molecules. A TNFbp(s) may also be covalently coupled to dinitrophenol (DNP) or trinitrophenol (TNP) and the resulting conjugates precipitated with anti-DNP or anti-TNP-IgM to form decameric conjugates.

In yet another embodiment, recombinant fusion proteins may also be produced wherein each recombinant chimeric molecule has a TNFbp(s) sequence amino-terminally or carboxy-terminally fused to all or part of the constant domains, but at least one constant domain, of the heavy or light chain of human immunoglobulin. For example, a chimeric TNFbp(s)/IgG1 (or IgG1/TNFbp(s)) fusion protein may be produced from a light chain-containing chimeric gene: a TNFbp(s)/human kappa light chain chimera (TNFbp (s)/Ck) or a human kappa light chain/TNFbp(s) chimera (Ck/TNFbp(s)); or a heavy chain-containing chimeric gene: a TNFbp(s)/human gamma-1 heavy chain chimera (TNFbp (s)/Cg-1) or a human gamma-1 heavy chain/TNFbp(s) chimera (Cg-1/TNFbp(s)). Following transcription and translation of a heavy-chain chimeric gene, or of a light chain-containing gene and a heavy-chain chimeric gene, the gene products may be assembled into a single chimeric molecule having a TNFbp(s) displayed bivalently. Additional details relating to the construction of such chimeric molecules are disclosed in U.S. Pat. 5,116,964, WO 89/09622, WO 91/16437 and EP 315062, the disclosures of which are hereby incorporated by reference.

In yet a further embodiment, recombinant fusion proteins may also be produced wherein each recombinant chimeric molecule has at least one TNFbp(s), as described herein, and at least a portion of the region 186–401 of osteoprotogerin, as described in European Patent Application No. 96309363.8, the disclosures of which are hereby incorporated by reference. Either the TNFbp(s) or the portion of osteoprotogerin may be at the amino-terminus or the carboxy-terminus of the chimeric molecule.

Synthesis of TNFbp(s)

The production of TNFbp(s) is described in further detail below. Such proteins may be prepared, for example, by recombinant techniques or by in vitro chemical synthesis.

Polynucleotides

Based upon the present description and using the universal codon table, one of ordinary skill in the art can readily determine all of the nucleic acid sequences which encode the amino acid sequence of the TNFbp(s).

Recombinant expression techniques conducted in accordance with the descriptions set forth below may be followed to produce each such polynucleotide and to express the encoded proteins. For example, by inserting a nucleic acid sequence which encodes a TNFbp(s) into an appropriate vector, one skilled in the art can readily produce large quantities of the desired nucleotide sequence. The sequences can then be used to generate detection probes or amplification primers. Alternatively, a polynucleotide encoding a TNFbp(s) can be inserted into an expression vector. By introducing the expression vector into an appropriate host, the desired protein may be produced in large amounts.

As further described herein, there are numerous host/vector systems available for the propagation of desired nucleic acid sequences and/or the production of the desired proteins. These include, but are not limited to, plasmid, viral and insertional vectors, and prokaryotic and eukaryotic hosts. One skilled in the art can adapt a host/vector system which is capable of propagating or expressing heterologous DNA to produce or express the sequences of the present invention.

Furthermore, it will be appreciated by those skilled in the art that, in view of the present disclosure, the nucleic acid sequences within the scope of the present invention include the nucleic acids of FIGS. **1** and **3**, as well as degenerate nucleic acid sequences thereof, nucleic acid sequences which encode variant(s) of the sTNFRs, and those nucleic acid sequences which hybridize to complements of the nucleic acids of FIGS. **1** and **3** under hybridization conditions, or equivalent conditions thereto, disclosed in the cDNA library screening section below.

Also provided by the present invention are recombinant DNA constructs involving vector DNA together with the DNA sequences encoding the desired proteins. In each such DNA construct, the nucleic acid sequence encoding a desired protein (with or without signal peptides) is in operative association with a suitable expression control or regulatory sequence capable of directing the replication and/or expression of the desired protein in a selected host.

Recombinant Expression

Preparation of Polynucleotides

A nucleic acid sequence encoding a TNFbp(s) can readily be obtained in a variety of ways including, without limitation, chemical synthesis, cDNA or genomic library screening, expression library screening and/or PCR amplification of cDNA. These methods and others which are useful for isolating such nucleic acid sequences are set forth in Sambrook et al. (1989), *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; by Ausubel et al. (1994), Current Protocols in *Molecular Biology,* Current Protocols Press; and by Berger and Kimmel (1987), *Methods in Enzymology: Guide to Molecular Cloning Techniques,* Vol. 152, Academic Press, Inc., San Diego, Calif., the disclosures of which are hereby incorporated by reference.

Chemical synthesis of a nucleic acid sequence which encodes a desired protein can be accomplished using methods well known in the art, such as those set forth by Engels et al. (1989), *Angew. Chem. Intl. Ed.,* 28:716–734 and Wells et al. (1985), *Gene,* 34:315, the disclosures of which is hereby incorporated by reference. These methods include, inter alia, the phosphotriester, phosphoramidite and

H-phosphonate methods of nucleic acid sequence synthesis. Large nucleic acid sequences, for example those larger than about 100 nucleotides in length, can be synthesized as several fragments. The fragments can then be ligated together to form a suitable nucleic acid sequence. A preferred method is polymer-supported synthesis using standard phosphoramidite chemistry.

Alternatively, a suitable nucleic acid sequence may be obtained by screening an appropriate cDNA library (i.e., a library prepared from one or more tissue sources believed to express the protein) or a genomic library (a library prepared from total genomic DNA). The source of the cDNA library is typically a tissue or cell source from any species that is believed to express a desired protein in reasonable quantities. The source of the genomic library may be any tissue or tissues from any mammalian or other species believed to harbor a gene encoding a desired protein.

Each hybridization medium can be screened for the presence of a DNA encoding a desired protein using one or more nucleic acid probes (oligonucleotides, cDNA or genomic DNA fragments that possess an acceptable level of homology to the cDNA or gene to be cloned) that will hybridize selectively with cDNA(s) or gene(s) present in the library. The probes typically used for such screening encode a small region of DNA sequence from the same or a similar species as the species from which the library is prepared. Alternatively, the probes may be degenerate, as discussed herein.

Hybridization is typically accomplished by annealing the oligonucleotide probe or cDNA to the clones under conditions of stringency that prevent non-specific binding but permit binding of those clones that have a significant level of homology with the probe or primer. Typical hybridization and washing stringency conditions depend in part on the size (i.e., number of nucleotides in length) of the cDNA or oligonucleotide probe and whether the probe is degenerate. The probability of identifying a clone is also considered in designing the hybridization medium (e.g., whether a cDNA or genomic library is being screened).

Where a DNA fragment (such as cDNA) is used as a probe, typical hybridization conditions include those as set forth in Ausubel et al. (1994), supra. After hybridization, the hybridization medium is washed at a suitable stringency, depending on several factors such as probe size, expected homology of probe to clone, the hybridization medium being screened, the number of clones being screened and the like.

Exemplary stringent hybridization conditions are hybridization in 6×SSC at 62–67° C., followed by washing in 0.1×SSC at 62–67° C. for approximately one hour. Alternatively, exemplary stringent hybridization conditions are hybridization at 45–55% formamide, 6×SSC at 40–45° C., followed by washing in 0.1×SSC at 62–67° C. for approximately one hour. Also included are DNA sequences which hybridize to the nucleic acid sequences set forth in FIGS. **1** and **3** under relaxed hybridization conditions and which encode a TNFbp(s). Examples of such relaxed stringency hybridization conditions are 6×SSC at 45–55° C. or hybridization with 30–40% formamide at 40–45° C., followed by washing in 1–2×SSC at 55° C. for approximately 30 minutes. See Maniatis et al. (1982), *Molecular Cloning* (A Laboratory Manual), Cold Spring Harbor Laboratory, pages 387 to 389, the disclosure of which is hereby incorporated by reference.

There are also exemplary protocols for stringent washing conditions where oligonucleotide probes are used to screen hybridization media. For example, a first protocol uses 6×SSC with 0.05 percent sodium pyrophosphate at a temperature of between about 35° C. and 63° C., depending on the length of the probe. For example, 14 base probes are washed at 35–40° C., 17 base probes at 45–50° C., 20 base probes at 52–57° C., and 23 base probes at 57–63° C. The temperature can be increased 2–3° C. where the background non-specific binding appears high. A second protocol uses tetramethylammonium chloride (TMAC) for washing. One such stringent washing solution is 3 M TMAC, 50 mM Tris-HCl, pH 8.0 and 0.2% SDS.

Another method for obtaining a suitable nucleic acid sequence encoding a TNFbp(s) is the polymerase chain reaction (PCR). In this method, cDNA is prepared from poly(A)+RNA or total RNA using the enzyme reverse transcriptase. Two primers, typically complementary to two separate regions of cDNA (oligonucleotides) encoding the desired protein, are then added to the cDNA along with a polymerase such as Taq polymerase and the polymerase amplifies the cDNA region between the two primers.

The oligonucleotide sequences selected as probes or primers should be of adequate length and sufficiently unambiguous so as to minimize the amount of non-specific binding that may occur during screening or PCR amplification. The actual sequence of the probes or primers is usually based on conserved or highly homologous sequences or regions. Optionally, the probes or primers can be fully or partially degenerate, i.e., can contain a mixture of probes/primers, all encoding the same amino acid sequence but using different codons to do so. An alternative to preparing degenerate probes is to place an inosine in some or all of those codon positions that vary by species. The oligonucleotide probes or primers may be prepared by chemical synthesis methods for DNA, as described herein.

Vectors

DNA encoding the desired proteins may be inserted into vectors for further cloning (amplification of the DNA) or for expression. Suitable vectors are commercially available or may be specifically constructed. The selection or construction of an appropriate vector will depend on (1) whether it is to be used for DNA amplification or for DNA expression, (2) the size of the DNA to be inserted into the vector and (3) the intended host cell to be transformed with the vector.

The vectors each typically involve a nucleic acid sequence which encodes a desired protein operatively linked to one or more of the following expression control or regulatory sequences capable of directing, controlling or otherwise effecting the expression of a desired protein by a selected host cell. Each vector contains various components, depending on its function (amplification of DNA or expression of DNA) and its compatibility with the intended host cell. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more selection or marker genes, a promoter, an enhancer element, a transcription termination sequence and the like. These components may be obtained from natural sources or be synthesized by known procedures.

Examples of suitable prokaryotic cloning vectors include bacteriophages such as lambda derivatives, or plasmids from *E. coli* (e.g. pBR322, col E1, pUC, the F-factor and Bluescript® plasmid derivatives (Stratagene, La Jolla, Calif.)). Other appropriate expression vectors, of which numerous types are known in the art for the host cells described below, can also be used for this purpose.

Signal Sequence

The nucleic acid encoding a signal sequence may be inserted 5' of the sequence encoding a desired protein, e.g,

it may be a component of a vector or it may be a part of a nucleic acid encoding a desired protein. The nucleic acids encoding the native signal sequences of the sTNFRs are known (EP 393 438, EP 422 339 and WO 96/28546, the disclosures of which are hereby incorporated by reference).

Origin of Replication

Expression and cloning vectors each generally include a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. In a cloning vector, this sequence is typically one that enables the vector to replicate independently of the host chromosomal DNA and includes an origin of replication or autonomously replicating sequence. Such sequences are well known. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, and various origins (e.g., Simian Virus 40 (SV40), polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication is not needed for mammalian expression vectors (for example, the SV40 origin is often used only because it contains the early promoter).

Selection Gene

The expression and cloning vectors each typically contain a selection gene. This gene encodes a "marker" protein necessary for the survival or growth of the transformed host cells when grown in a selective culture media. Host cells that are not transformed with the vector will not contain the selection gene and, therefore, will not survive in the culture media. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate or tetracycline; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from the culture media.

Other selection genes may be used to amplify the genes to be expressed. Amplification is the process wherein genes which are in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of suitable selectable markers for mammalian cells include dihydrofolate reductase (DHFR) and thymidine kinase. The cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of the marker being present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the media is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the desired protein. As a result, increased quantities of the desired protein are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture media that contains methotrexate, a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is used is the Chinese hamster ovary cell line deficient in DHFR activity (Urlaub and Chasin (1980), *Proc. Natl. Acad. Sci., USA,* 77(7):4216–4220, the disclosure of which is hereby incorporated by reference). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene and, concomitantly, multiple copies of other DNA present in the expression vector, such as the DNA encoding a desired protein.

Promoter

Expression and cloning vectors each will typically contain a promoter that is recognized by the host organism and is operably linked to a nucleic acid sequence encoding the desired protein. A promoter is an untranslated sequence located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that controls the transcription and translation of a particular nucleic acid sequence. A promoter may be conventionally grouped into one of two classes, inducible promoters and constitutive promoters. An inducible promoter initiates increased levels of transcription from DNA under its control in response to some change in culture conditions, such as the presence or absence of a nutrient or a change in temperature. A large number of promoters, recognized by a variety of potential host cells, are well known. A promoter may be operably linked to DNA encoding a desired protein by removing the promoter from the source DNA by restriction enzyme digestion and inserting the desired promoter sequence. The native promoter sequences of sTNFRs may be used to direct amplification and/or expression of the DNA encoding a desired protein. A heterologous promoter is preferred, however, if it permits greater transcription and higher yields of the expressed protein as compared to the native promoter and if it is compatible with the host cell system that has been selected for use. For example, any one of the native promoter sequences of other NGF/TNF receptor family members may be used to direct amplification and/or expression of the DNA encoding a desired protein.

Promoters suitable for use with prokaryotic hosts include the beta-lactamase and lactose promoter systems; alkaline phosphatase; a tryptophan (trp) promoter system; a bacterial luminescence (luxR) gene system and hybrid promoters such as the tac promoter. Other known bacterial promoters are also suitable. Their nucleotide sequences have been published, thereby enabling one skilled in the art to ligate each selected sequence to the desired DNA sequence using linkers or adaptors as needed to supply any required restriction sites.

Suitable promoter sequences for use with yeast hosts are also well known in the art. Suitable promoters for use with mammalian host cells are well known and include those obtained from the genomes of viruses such as polyoma virus, fowlpox virus; adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and, most preferably, SV40. Other suitable mammalian promoters include heterologous mammalian promoters, e.g., heat-shock promoters and the actin promoter.

Enhancer Element

The expression and cloning vectors each will typically contain an enhancer sequence to increase the transcription by higher eukaryotes of a DNA sequence encoding a desired protein. Enhancers are cis-acting elements of DNA, usually from about 10–300 bp in length, that act on the promoter to increase its transcription. Enhancers are relatively orientation and position independent. They have been found 5' and 3' to the transcription unit. Yeast enhancers are advantageously used with yeast promoters. Several enhancer sequences available from mammalian genes are known (e.g., globin, elastase, albumin, alpha-feto-protein and insulin). Additionally, viral enhancers such as the SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer and adenovirus enhancers are exemplary enhancing elements for the activation of eukaryotic promoters. While an enhancer may be spliced into a vector at a position 5' or 3' to a DNA encoding a desired protein, it is typically located at a site 5' from the promoter.

Transcription Termination

Expression vectors used in eukaryotic host cells each will typically contain a sequence necessary for the termination of transcription and for stabilizing the mRNA. Such sequences

are commonly available from the 5' and occasionally 3' untranslated regions of eukaryotic DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding a desired protein.

Vector Construction

The construction of a suitable vector containing one or more of the herein-listed components (together with the desired coding sequence) may be accomplished by standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored and religated in the desired order to generate the required vector. To confirm that the correct sequence has been constructed, the ligation mixture may be used to transform *E. coli,* and successful transformants may be selected by known techniques as described herein. Quantities of the vector from the transformants are then prepared, analyzed by restriction endonuclease digestion and/or sequenced to confirm the presence of the desired construct.

A vector that provides for the transient expression of DNA encoding a desired protein in mammalian cells may also be used. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of the desired protein encoded by the expression vector. Each transient expression system, comprising a suitable expression vector and a host cell, allows for the convenient positive identification of proteins encoded by cloned DNAs, as well as for the rapid screening of such proteins for desired biological or physiological properties.

Host Cells

Any of a variety of recombinant host cells, each of which contains a nucleic acid sequence for use in expressing a desired protein, is also provided by the present invention. Exemplary prokaryotic and eukaryotic host cells include bacterial, mammalian, fungal, insect, yeast or plant cells.

Prokaryotic host cells include, but are not limited to, eubacteria such as Gram-negative or Gram-positive organisms (e.g., *E. coli* (HB101, DH5a, DH10, and MC1061); Bacilli spp. such as *B. subtilis;* Pseudomonas spp. such as *P. aeruginosa;* Streptomyces spp.; Salmonella spp. such as *S. typhimurium;* or Serratia spp. such as *S. marcescans.* In a specific embodiment, a desired protein may be expressed in *E. coli.*

In addition to prokaryotic host cells, TNFbp(s) may be expressed in glycosylated form by any one of a number of suitable host cells derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture might be used, whether such culture involves vertebrate or invertebrate cells, including plant and insect cells. Eukaryotic microbes such as filamentous fungi or yeast may be suitable hosts for the expression of a desired protein. *Saccharomyces cerevisiae,* or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms, but a number of other genera, species and strains are well known and commonly available.

Vertebrate cells may be used, as the propagation of vertebrate cells in culture (tissue culture) is a well-known procedure. Examples of useful mammalian host cell lines include, but are not limited to, monkey kidney CV1 line transformed by SV40 (COS-7), human embryonic kidney line (293 cells or 293 cells subcloned for growth in suspension culture), baby hamster kidney cells and Chinese hamster ovary cells. Other suitable mammalian cell lines include, but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, and BHK or HaK hamster cell lines. In a specific embodiment, a desired protein may be expressed in COS cells or in baculovirus cells.

A host cell may be transfected and preferably transformed with a desired nucleic acid under appropriate conditions permitting expression of the nucleic acid. The selection of suitable host cells and methods for transformation, culture, amplification, screening and product production and purification are well known in the art (Gething and Sambrook (1981), *Nature,* 293:620–625 or, alternatively, Kaufman et al. (1985), *Mol. Cell. Biol.,* 5(7):1750–1759, or U.S. Pat. No. 4,419,446, the disclosures of which are hereby incorporated by reference). For example, for mammalian cells without cell walls, the calcium phosphate precipitation method may be used. Electroporation, micro-injection and other known techniques may also be used.

It is also possible that a desired protein may be produced by homologous recombination or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding a desired protein. Homologous recombination is a technique originally developed for targeting genes to induce or correct mutations in transcriptionally-active genes (Kucherlapati (1989), *Prog. in Nucl. Acid Res. and Mol. Biol.,* 36:301, the disclosure of which is hereby incorporated by reference). The basic technique was developed as a method for introducing specific mutations into specific regions of the mammalian genome (Thomas et al. (1986), *Cell,* 44:419–428; Thomas and Capecchi (1987), *Cell,* 51:503–512 and Doetschman et al. (1988), *Proc. Natl. Acad. Sci.,* 85:8583–8587, the disclosures of which are hereby incorporated by reference) or to correct specific mutations within defective genes (Doetschman et al. (1987), *Nature,* 330:576–578, the disclosure of which is hereby incorporated by reference). Exemplary techniques are described in U.S. Pat. No. 5,272,071; WO 92/01069; WO 93/03183; WO 94/12650 and WO 94/31560, the disclosures of which are hereby incorporated by reference.

Culturing the Host Cells

The method for culturing each of the one or more recombinant host cells for production will vary depending upon many factors and considerations; the optimum production procedure for a given situation will be apparent to those skilled in the art through minimal experimentation. Such recombinant host cells are cultured in a suitable media and the expressed protein is then optionally recovered, isolated and purified from the culture media (or from the cell, if expressed intracellularly) by appropriate means known to those skilled in the art.

Specifically, each of the recombinant cells used to produce a desired protein may be cultured in a culture media suitable for inducing promoters, selecting suitable recombinant host cells or amplifying the gene encoding the desired protein. The culture media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as gentamicin), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or another energy source. Other supplements may also be included, at appropriate concentrations, as will be appreciated by those skilled in the art. Suitable culture conditions, such as temperature, pH and the like, are also well known to those skilled in the art for use with the selected host cells.

The resulting expression product may then be purified to near homogeneity by using procedures known in the art.

Exemplary purification techniques are taught in EP 393 438 and EP 422 339, the disclosures of which are hereby incorporated by reference.

Pharmaceutical Compositions

The present invention encompasses pharmaceutical preparations each containing therapeutically- or prophylactically-effective amounts of a TNFbp(s) or a chemically-modified derivative thereof (collectively, "TNFbp product(s)") in admixture with a vehicle. The vehicle preferably includes one or more pharmaceutically and physiologically acceptable formulation materials in admixture with the TNFbp product(s).

The primary solvent in a vehicle may be either aqueous or non-aqueous in nature. In addition, the vehicle may contain pharmaceutically acceptable excipients for modifying or maintaining the pH preferably between 5–6.5, and more preferably between 5.5–6.0 (e.g., buffers such as citrates or phosphates, and amino acids such glycine); viscosity; clarity; color; sterility; stability (e.g., sucrose and sorbitol); odor; rate of dissolution (e.g., solubilizers or solubilizing agents such as alcohols, polyethylene glycols and sodium chloride); rate of release; as well as bulking agents for lyophilized formulation (e.g., mannitol and glycine); surfactants (e.g., polysorbate 20, polysorbate 80, triton and pluronics); antioxidants (e.g., sodium sulfite and sodium hydrogen-sulfite); preservatives (e.g., benzoic acid and salicylic acid); flavoring and diluting agents; emulsifying agents; suspending agents; solvents; fillers; delivery vehicles and other pharmaceutical adjuvants and/or excipients. Other effective administration forms such as parenteral slow-release formulations, inhalant mists, orally-active formulations, or suppositories are also envisioned. The composition may also involve particulate preparations of polymeric compounds such as bulk erosion polymers (e.g., poly(lactic-co-glycolic acid) (PLGA) copolymers, PLGA polymer blends, block copolymers of PEG, and lactic and glycolic acid, poly(cyanoacrylates)); surface erosion polymers (e.g., poly(anhydrides) and poly(ortho esters)); hydrogel esters (e.g., pluronic polyols, poly(vinyl alcohol), poly (vinylpyrrolidone), maleic anhydride-alkyl vinyl ether copolymers, cellulose, hyaluronic acid derivatives, alginate, collagen, gelatin, albumin, and starches and dextrans) and composition systems thereof; or preparations of liposomes or microspheres. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. The optimal pharmaceutical formulation for a desired protein will be determined by one skilled in the art depending upon the route of administration and desired dosage. Exemplary pharmaceutical compositions are disclosed in *Remington's Pharmaceutical Sciences,* 18th Ed. (1990), Mack Publishing Co., Easton, Pa. 18042, pages 1435– 1712; Gombotz and Pettit (1995), *Bioconjugate Chem.,* 6:332–351; Leone-Bay, et al. (1995), *Journal of Medicinal Chemistry,* 38:4263–4269; Haas, et al. (1995), *Clinical Immunology and Immunopathology,* 76(1):93; WO 94/06457; WO 94/21275; FR 2706772 and WO 94/21235, the disclosures of which are incorporated herein by reference.

Specific sustained release compositions are available from the following suppliers: Depotech (Depofoam™, a multivesicular liposome) and Alkermes (ProLease™, a PLGA microsphere). Exemplary forms of hyaluronan are disclosed in Peyron and Balazs (1974), *Path. Biol.,* 22(8) :731–736; Isdale et al. (1991), *J. Drug Dev.,* 4(2):93–99; Larsen et al. (1993), *Journal of Biomedical Materials Research,* 27:1129–1134; Namiki, et al. (1982), *International Journal of Clinical Pharmacology, Therapy and Toxicology,* 20(11):501–507; Meyer et al. (1995), *Journal of Controlled Release,* 35:67–72; Kikuchi et al. (1996), *Osteoarthritis and Cartilage,* 4:99–110; Sakakibara et al. (1994), *Clinical Orthopaedics and Related Research,* 299:282–292; Meyers and Brandt (1995), 22(9):1732–1739; Laurent et al. (1995), *Acta Orthop Scand,* 66(266):116–120; Cascone et al. (1995), *Biomaterials,* 16(7):569–574; Yerashalmi et al. (1994), *Archives of Biochemistry and Biophysics,* 313(2):267–273; Bernatchez et al. (1993), *Journal of Biomedical Materials Research,* 27(5):677–681; Tan et al. (1990), *Australian Journal of Biotechnology,* 4(1) :38–43; Gombotz and Pettit (1995), *Bioconjugate Chem.,* 6:332–351; U.S. Pat. Nos. 4,582,865, 4,605,691, 4,636,524, 4,713,448, 4,716,154, 4,716,224, 4,772,419, 4,851,521, 4,957,774, 4,863,907, 5,128,326, 5,202,431, 5,336,767, 5,356,883; European Patent Application Nos. 0 507 604 A2 and 0 718 312 A2; and WO 96/05845, the disclosures of which are hereby incorporated by reference. Specific hyaluronan compositions are available from the following suppliers: BioMatrix, Inc. Ridgefield, N.J. (Synvisc™, a 90:10 mixture of a hylan fluid and hylan gel); Fidia S.p.A., Abano Terme, Italy (Hyalgan™, the sodium salt of a rooster comb-derived hyaluronic acid (~500,000 to ~700,000 MW)); Kaken Pharmaceutical Co., Ltd., Tokyo, Japan (Artz™, a 1% solution of a rooster-comb derived hyaluronic acid, ~700,000 MW); Pharmacia AB, Stockholm, Sweden (Healon™, a rooster-comb derived hyaluronic acid, ~4×10^6 MW); Genzyme Corporation, Cambridge, Mass. (Surgicoat™, a recombinant hyaluronic acid); Pronova Biopolymer, Inc. Portsmouth, N.H. (Hyaluronic Acid FCH, a high molecular weight (e.g., ~$1.5–2.2\times10^6$ MW) hyaluronic acid prepared from cultures of *Streptococcus zooepidemicus;* Sodium Hyaluronate MV, ~$1.0–1.6\times10^6$ MW and Sodium Hyaluronate LV, ~$1.5–2.2\times10^6$ MW); Calbiochem-Novabiochem AB, Lautelfingen, Switzerland (Hyaluronic Acid, sodium salt (1997 company catalog number 385908) prepared from Streptococcus sp.); Intergen Company, Purchase, N.Y. (a rooster-comb derived hyaluronic acid, >1×10^6 MW); Diosynth Inc., Chicago, Ill.; Amerchol Corp., Edison, N.J. and Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan.

Once the pharmaceutical composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or a dehydrated or lyophilized powder. Such compositions each may be stored either in a ready-to-use form or in a form (e.g., lyophilized) requiring reconstitution prior to administration. In a specific embodiment, the present invention is directed to kits for producing a single-dose administration unit. The kits may each contain both a first container having a dried protein and a second container having an aqueous formulation. Kits included within the scope of this invention are single and multi-chambered pre-filled syringes; exemplary pre-filled syringes (e.g., liquid syringes, and lyosyringes such as Lyo-Ject®, a dual-chamber pre-filled lyosyringe) are available from Vetter GmbH, Ravensburg, Germany.

Uses

TNFbp product(s) may be useful as research reagents and as therapeutic and diagnostic agents. Thus the TNFbp product(s) may be used in in vitro and/or in vivo diagnostic assays to quantify the amount of native TNFR-I, sTNFR-I, TNFR-II or sTNFR-II in a tissue or organ sample or to determine and/or isolate cells which express TNF (Scallon et al. (1995), supra). In assays of tissues or organs there will be less radioactivity from an ^{125}I-TNFbp product(s) binding to TNF, as compared to a standardized binding curve of an ^{125}I-TNFbp product(s), due to unlabeled native sTNFR-I or

sTNFR-II binding to TNF. Similarly, the use of an ^{125}I-TNFbp product(s) may be used to detect the presence of TNF in various cell types.

This invention also contemplates the use of TNFbp product(s) in the generation of antibodies and the resultant antibodies (specifically including those which also bind to native sTNFR-I or sTNFR-II). Antibodies can be developed which bind to TNFbp product(s). One of ordinary skill in the art can use well-known published procedures to obtain monoclonal, polyclonal antibodies or recombinant antibodies which specifically recognize and bind to the various proteins encoded by the amino acid sequences of the present invention. Such antibodies may then be used to purify and characterize the native sTNFR-I and native sTNFR-II, or to quantify the number of TNFR-I or TNFR-II expressed on a cell surface.

The present invention also relates to methods for the treatment of certain diseases and medical conditions (many of which can be characterized as inflammatory diseases) that are mediated by TNF, as well as the related sequela and symptoms associated therewith. A non-exclusive list of acute and chronic TNF-mediated diseases includes but is not limited to the following: cachexia/anorexia; cancer (e.g., leukemias); chronic fatigue syndrome; depression; diabetes (e.g., juvenile onset Type 1 and diabetes mellitus); fibromyelgia or analgesia; graft versus host rejection; hyperalgesia; inflammatory bowel disease; ischemic, including cerebral ischemia (brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration); lung diseases (e.g., adult respiratory distress syndrome and pulmonary fibrosis); multiple sclerosis; neuroinflammatory diseases; ocular diseases; pain; pancreatitis; pulmonary fibrosis; reperfusion injury; rheumatic diseases (e.g., rheumatoid arthritis, osteoarthritis, juvenile (rheumatoid) arthritis, seronegative polyarthritis, ankylosing spondylitis, Reiter's syndrome and reactive arthritis, psoriatic arthritis, enteropathic arthritis, polymyositis, dermatomyositis, scleroderma, systemic sclerosis, vasculitis, cerebral vasculitis, Lyme disease, staphylococcal-induced ("septic") arthritis, Sjögren's syndrome, rheumatic fever, polychondritis and polymyalgia rheumatica and giant cell arteritis); septic shock; side effects from radiation therapy; systemic lupus erythematous; temporal mandibular joint disease; thyroiditis; tissue transplantation or an inflammatory condition resulting from strain, sprain, cartilage damage, trauma, orthopedic surgery, infection or other disease process.

The TNFbp product(s) may be administered to a patient in therapeutically effective amounts for the prevention or treatment of TNF-mediated diseases, including rheumatic diseases. The term "patient" is intended to encompass animals (e.g., cats, dogs and horses) as well as humans.

TNFbp product(s) may be administered via topical, enteral or parenteral administration including, without limitation, infusion, intraarterial, intraarticular, intracapsular, intracardiac, intradermal, intramuscular, intraorbital, intrathecal, intravenous, intraperitoneal, intraspinal, intrasternal injection, intraventricular, subcutaneous, subcuticular, subcapsular, subarachnoid and transtracheal. TNFbp product(s) may also be administered via oral administration or be administered through mucus membranes, that is, buccally, intranasally, rectally or sublingually for systemic delivery.

It is preferred that TNFbp product(s) be administered via intraarticular, intramuscular, intravenous or subcutaneous injection. Additionally, TNFbp product(s) may be administered by continuous infusion (e.g., constant or intermittent implanted or external infusion flow-modulating devices) so as to continuously provide the desired level of TNFbp product(s) in the blood for the duration of the administration. This is may be accomplished by means of a mini-pump, such as an osmotic mini-pump. In these ways, one can be assured that the amount of drug is maintained at the desired level and one can take blood samples and monitor the amount of drug in the bloodstream. Various pumps are commercially available, for example, from suppliers such as MiniMed Inc., Sylmar, Calif. (e.g., MT507) and Alza Corp., Palo Alto, Calif. (e.g. Alzet osmotic pump, model 2MLI).

It is also contemplated that other modes of continuous or near-continuous dosing may be practiced. For example, chemical derivatization may result in sustained release forms of the protein which have the effect of continuous presence in the blood stream, in predictable amounts based on a determined dosage regimen.

Modes of using TNFbp product(s) for the treatment of TNF-mediated diseases, including rheumatic diseases (e.g., osteoarthritis, psoriatic arthritis and rheumatoid arthritis), are set forth in European Patent Application 567566, the teachings of which are hereby incorporated by reference. By way of example but not limitation, in one specific embodiment, TNFbp product(s) may be administered intra-articularly for the treatment of rheumatoid arthritis and osteoarthritis. By way of example but not limitation in another specific embodiment, TNFbp product(s) may be administered subcutaneously or intramuscularly for the treatment of rheumatoid arthritis, inflammatory bowel disease, cachexia/anorexia or multiple sclerosis. By way of example but not limitation, in a still further specific embodiment TNFbp product(s) may be administered intravenously for the treatment of brain injury as a result of trauma, epilepsy, hemorrhage or stroke; or administered intraventricularly for the treatment of brain injury as a result of trauma. A specific mode for the treatment of arthritis includes: (1) a single intraarticular injection of a TNFbp product(s) given periodically as needed to prevent or remedy the flare-up of arthritis and (2) periodic subcutaneous injections of TNFbp product(s). In another specific embodiment, a TNFbp product(s) may be administered in the treatment of septic shock. The initiation of treatment for septic shock should begin as soon as possible after septicemia or the chance of septicemia is diagnosed. For example, treatment may be begun immediately following surgery or an accident or any other event that may carry the risk of initiating septic shock. Preferred modes for the treatment of adult respiratory distress syndrome include: (1) single or multiple intratracheal administrations of a TNFbp product(s) and (2) bolus or continuous intravenous infusion of a TNFbp product(s).

In another embodiment, cell therapy is also contemplated, e.g., implantation of cells producing a TNFbp product(s). This embodiment of the present invention may include implanting into patients cells which are capable of synthesizing and secreting a TNFbp product(s). Such cells producing a TNFbp product(s) may be cells which do not normally produce a TNFbp product(s) but which have been modified to produce a TNFbp product(s). The cells also may be cells whose ability to produce a TNFbp product(s) have been augmented by transformation with a polynucleotide suitable for the expression and secretion of a TNFbp product(s). In order to minimize a potential immunological reaction in patients by administering a TNFbp product(s) of a foreign species, it is preferred that the cells be of the same species as the patient (e.g., human) or that the cells be encapsulated with material that provides a barrier against immune recognition, or that cells be placed into an immunologically

privileged anatomical location, such as in the testis, eye or central nervous system.

Human or non-human animal cells may be implanted into patients in biocompatible, semi-permeable polymeric enclosures or membranes to allow release of a TNFbp product(s), but to prevent destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissue. Alternatively, the patient's own cells, transformed ex vivo to produce a TNFbp product(s), may be implanted directly into the patient without such encapsulation. The methodology for the membrane encapsulation of living cells is familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished.

In yet another embodiment, in vivo gene therapy is also envisioned, wherein a nucleic acid sequence encoding a TNFbp product(s) is introduced directly into a patient. For example, a nucleic acid sequence encoding a TNFbp product (s) is introduced into target cells via local injection of a nucleic acid construct, with or without an appropriate delivery vector, such as an adeno-associated virus vector. Alternative viral vectors include but are not limited to retrovirus, adenovirus, herpes simplex virus and papilloma virus vectors. Physical transfer may be achieved in vivo by local injection of the desired nucleic acid construct or other appropriate delivery vector containing the desired nucleic acid sequence, liposome-mediated transfer, direct injection (naked DNA), receptor-mediated transfer (ligand-DNA complex) or microparticle bombardment (gene gun).

Exemplary cell and gene therapy techniques are disclosed in U.S. Pat. Nos. 4,892,538; 5,011,472; 5,106,627; DE 4219626, WO 94/20517 and 96/22793, the disclosures of which are hereby incorporated by reference.

Regardless of the manner of administration, the treatment of a TNF-mediated disease requires a dose or total dose regimen of a TNFbp product(s) effective to reduce or alleviate symptoms of the disease. Factors in determining the appropriate dosage or total dose regimen can include the disease or condition to be treated or prevented, the severity of the disease, the route of administration, and the age, sex and medical condition of the patient.

Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those skilled in the art, especially in light of the dosage information and assays disclosed herein. The frequency of dosing also depends on the pharmacokinetic parameters of the TNFbp product(s) in the formulation used. The TNFbp product(s) may be administered once, or in cases of severe and prolonged disorders, administered daily in less frequent doses or administered with an initial bolus dose followed by a continuous dose or sustained delivery. It is also contemplated that other modes of continuous or near-continuous dosing may be practiced. For example, chemical derivatization may result in sustained release forms which have the effect of a continuous presence in the bloodstream, in predictable amounts based on a determined dosage or total dosage regimen. The dosage or total dose regimen can also be determined through the use of known assays for determining dosages used in conjunction with appropriate dose-response data.

When administered parenterally, each unit dose, for example, may be up to 10 mg, generally up to 15 mg and more generally up to 20 mg. When administered into an articular cavity, the pharmaceutical composition is preferably administered as a single injection from, for example, a 3 to 10 ml syringe containing a dose, for example, of between about 5 mg/ml to 10 mg/ml TNFbp product(s) dissolved in isotonic phosphate buffered saline. The preparation may be administered into an articular cavity at a frequency, for example, of once every 7 to 10 days. In such a manner, the administration is continuously conducted, for example, 4 to 5 times while varying the dose if necessary.

As contemplated by the present invention, a TNFbp product(s) may be administered as an adjunct to other therapy and also with other pharmaceutical formulations suitable for the indication being treated. A TNFbp product(s) and any of one or more additional therapies or pharmaceutical formulations may be administered separately or in combination.

In a specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with one or more additional TNF inhibitors for the treatment of TNF-mediated diseases, including acute and chronic inflammation. TNF inhibitors include compounds and proteins which block in vivo synthesis or extracellular release of TNF, including the following compounds.

Additional TNF inhibitors include anti-TNF antibodies (e.g., MAK 195F Fab antibody (Holler et al. (1993), 1st International Symposium on Cytokines in Bone Marrow Transplantation, 147; CDP 571 anti-TNF monoclonal antibody (Rankin et al. (1995), *British Journal of Rheumatology,* 34:334–342, the disclosure of which is incorporated by reference); BAY X 1351 murine anti-tumor necrosis factor monoclonal antibody (Kieft et al. (1995), 7th European Congress of Clinical Microbiology and Infectious Diseases, 9, the disclosure of which is incorporated by reference); CenTNF cA2 anti-TNF monoclonal antibody (Elliott et al. (1994), *Lancet,* 344:1125–1127 and Elliott et al. (1994), *Lancet,* 344:1105–1110, the disclosures of which are incorporated by reference).

In a specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with secreted or soluble human fas antigen or recombinant versions thereof (WO 96/20206 and Mountz et al., *J. Immunology,* 155:4829–4837; and EP 510 691, the disclosures of which are hereby incorporated by reference). WO 96/20206 discloses secreted human fas antigen (native and recombinant, including an Ig fusion protein), methods for isolating the genes responsible for coding the soluble recombinant human fas antigen, methods for cloning the gene in suitable vectors and cell types, and methods for expressing the gene to produce the inhibitors. EP 510 691 teaches DNAs coding for human fas antigen, including soluble fas antigen, vectors expressing for said DNAs and transformants transfected with the vector. When administered parenterally, doses of a secreted or soluble fas antigen fusion protein each are generally from about 1 micrograms/kg to about 100 micrograms/kg.

In a specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more interleukin-1 inhibitors for the treatment of TNF-mediated diseases, including acute and chronic inflammation such as cachexia/anorexia; chronic fatigue syndrome, depression; diabetes (e.g., juvenile onset Type 1 and diabetes mellitus); fibromyelgia or analgesia; graft versus host rejection; hyperalgesia, inflammatory bowel disease; ischemic injury, including cerebral ischemia (e.g., brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration); lung diseases (e.g., ARDS and pulmonary fibrosis); multiple sclerosis, ocular diseases; pain; pancreatitis, reperfusion

injury; rheumatic diseases (e.g., rheumatoid arthritis, osteoarthritis, juvenile (rheumatoid) arthritis, seronegative polyarthritis, ankylosing spondylitis, Reiter's syndrome and reactive arthritis, psoriatic arthritis, enteropathic arthritis, polymyositis, dermatomyositis, scleroderma, systemic sclerosis, vasculitis, cerebral vasculitis, Lyme disease, staphylococcal-induced ("septic") arthritis, Sjögren's syndrome, rheumatic fever, polychondritis and polymyalgia rheumatica and giant cell arteritis); septic shock; side effects from radiation therapy; temporal mandibular joint disease; tumor metastasis; or an inflammatory condition resulting from strain, sprain, cartilage damage, trauma, orthopedic surgery, infection or other disease processes. Classes of interleukin-1 inhibitors include interleukin-1 receptor antagonists (any compound capable of specifically preventing activation of cellular receptors to IL-1) such as IL-1ra, as described below; anti-IL-1 receptor monoclonal antibodies (e.g., EP 623674), the disclosure of which is hereby incorporated by reference; IL-1 binding proteins such as soluble IL-1 receptors (e.g., U.S. Pat. Nos. 5,492,888, 5,488, 032, and 5,464,937, 5,319,071, and 5,180,812, the disclosures of which are hereby incorporated by reference); anti-IL-1 monoclonal antibodies (e.g., WO 9501997, WO 9402627, WO 9006371, U.S. Pat. No. 4,935,343, EP 364778, EP 267611 and EP 220063, the disclosures of which are hereby incorporated by reference); IL-1 receptor accessory proteins (e.g., WO 96/23067, the disclosure of which is hereby incorporated by reference), and other compounds and proteins which block in vivo synthesis or extracellular release of IL-1.

Interleukin-1 receptor antagonist (IL-1ra) is a human protein that acts as a natural inhibitor of interleukin-1. Preferred receptor antagonists, as well as methods of making and methods of using thereof, are described in U.S. Pat. No. 5,075,222; WO 91/08285; WO 91/17184; AU 9173636; WO 92/16221; WO93/21946; WO 94/06457; WO 94/21275; FR 2706772; WO 94/21235; DE 4219626; WO 94/20517; WO 96/22793 and WO 97/28828 the disclosures of which are incorporated herein by reference. The proteins include glycosylated as well as non-glycosylated IL-1 receptor antagonists.

Specifically, three preferred forms of IL-1ra (IL-1raα, IL-1raβ and IL-1rax), each being derived from the same DNA coding sequence, are disclosed and described in U.S. Pat. No. 5,075,222. Methods for producing IL-1 inhibitors, particularly IL-1ras, are also disclosed in the 5,075,222 patent. In a specific embodiment, an IL-1ra contains an N-terminal methionyl group as a consequence of expression in *E. coli*. The present invention also includes modified IL-1ras. The modified IL-1ras include, for example, muteins of such inhibitors in which a cysteine residue is substituted for an amino acid at one or more sites in the amino acid sequence of a naturally-occurring inhibitor. Such muteins may then be site-selectively reacted with functionalized polyethylene glycol (PEG) units or other sulfhydryl-containing polyethers to create IL-1ra PEG species. WO 92/16221 discloses a number of modified IL-1ra species and methods of making such PEG modified inhibitors.

An additional class of interleukin-1 inhibitors includes compounds capable of specifically prventing activation of cellular receptors to IL-1. Such compounds include IL-1 binding proteins, such as soluble receptors and monoclonal antibodies. Such compounds also include monoclonal antibodies to the receptors.

A further class of interleukin-1 inhibitors includes compounds and proteins which block in vivo synthesis and/or extracellular release of IL-1. Such compounds include agents which affect transcription of IL-1 genes or processing of IL-1 preproteins.

Present treatment of TNF-mediated diseases, including acute and chronic inflammation such as rheumatic diseases includes the use of first line drugs for control of pain and inflammation classified as non-steroidal, anti-inflammatory drugs (NSAIDs). Secondary treatments include corticosteroids, slow acting antirheumatic drugs (SAARDs) or disease modifying (DM) drugs. Information regarding the following compounds can be found in The Merck Manual of Diagnosis and Therapy, Sixteenth Edition, Merck, Sharp & Dohme Research Laboratories, Merck & Co., Rahway, N.J. (1992) and in Pharmaprojects, PJB Publications Ltd.

In a specific embodiment, the present invention is directed to the use of a TNFbp product(s) and any of one or more NSAIDs for the treatment of TNF-mediated diseases, including acute and chronic inflammation such as rheumatic diseases and graft versus host disease. NSAIDs owe their anti-inflammatory action, at least in part, to the inhibition of prostaglandin synthesis (Goodman and Gilman in "The Pharmacological Basis of Therapeutics," MacMillan 7th Edition (1985)). NSAIDs can be characterized into nine groups: (1) salicylic acid derivatives; (2) propionic acid derivatives; (3) acetic acid derivatives; (4) fenamic acid derivatives; (5) carboxylic acid derivatives; (6) butyric acid derivatives; (7) oxicams; (8) pyrazoles and (9) pyrazolones.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more salicylic acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. Such salicylic acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof comprise: acetaminosalol, aloxiprin, aspirin, benorylate, bromosaligenin, calcium acetylsalicylate, choline magnesium trisalicylate diflusinal, etersalate, fendosal, gentisic acid, glycol salicylate, imidazole salicylate, lysine acetylsalicylate, mesalamine, morpholine salicylate, 1-naphthyl salicylate, olsalazine, parsalmide, phenyl acetylsalicylate, phenyl salicylate, salacetamide, salicylamide O-acetic acid, salsalate and sulfasalazine. Structurally related salicylic acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more propionic acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. The propionic acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof comprise: alminoprofen, benoxaprofen, bucloxic acid, carprofen, dexindoprofen, fenoprofen, flunoxaprofen, fluprofen, flurbiprofen, furcloprofen, ibuprofen, ibuprofen aluminum, ibuproxam, indoprofen, isoprofen, ketoprofen, loxoprofen, miroprofen, naproxen, oxaprozin, piketoprofen, pimeprofen, pirprofen, pranoprofen, protizinic acid, pyridoxiprofen, suprofen, tiaprofenic acid and tioxaprofen. Structurally related propionic acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more acetic acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. The acetic acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof comprise: acemetacin, alclofenac, amfenac,

bufexamac, cinmetacin, clopirac, delmetacin, diclofenac sodium, etodolac, felbinac, fenclofenac, fenclorac, fenclozic acid, fentiazac, furofenac, glucametacin, ibufenac, indomethacin, isofezolac, isoxepac, lonazolac, metiazinic acid, oxametacin, oxpinac, pimetacin, proglumetacin, sulindac, talmetacin, tiaramide, tiopinac, tolmetin, zidometacin and zomepirac. Structurally related acetic acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more fenamic acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. The fenamic acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof comprise: enfenamic acid, etofenamate, flufenamic acid, isonixin, meclofenamic acid, meclofenamate sodium, medofenamic acid, mefanamic acid, niflumic acid, talniflumate, terofenamate, tolfenamic acid and ufenamate. Structurally related fenamic acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more carboxylic acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. The carboxylic acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof which can be used comprise: clidanac, diflunisal, flufenisal, inoridine, ketorolac and tinoridine. Structurally related carboxylic acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more butyric acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. The butyric acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof comprise: bumadizon, butibufen, fenbufen and xenbucin. Structurally related butyric acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more oxicams, prodrug esters or pharmaceutically acceptable salts thereof. The oxicams, prodrug esters and pharmaceutically acceptable salts thereof comprise: droxicam, enolicam, isoxicam, piroxicam, sudoxicam, tenoxicam and 4-hydroxyl-1,2-benzothiazine 1,1-dioxide 4-(N-phenyl)-carboxamide. Structurally related oxicams having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more pyrazoles, prodrug esters or pharmaceutically acceptable salts thereof. The pyrazoles, prodrug esters and pharmaceutically acceptable salts thereof which may be used comprise: difenamizole and epirizole. Structurally related pyrazoles having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more pyrazolones, prodrug esters or pharmaceutically acceptable salts thereof. The pyrazolones, prodrug esters and pharmaceutically acceptable salts thereof which may be used comprise: apazone, azapropazone, benzpiperylon, feprazone, mofebutazone, morazone, oxyphenbutazone, phenylbutazone, pipebuzone, propylphenazone, ramifenazone, suxibuzone and thiazolinobutazone. Structurally related pyrazalones having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more of the following NSAIDs: ε-acetamidocaproic acid, S-adenosylmethionine, 3-amino-4-hydroxybutyric acid, amixetrine, anitrazafen, antrafenine, bendazac, bendazac lysinate, benzydamine, beprozin, broperamole, bucolome, bufezolac, ciproquazone, cloximate, dazidamine, deboxamet, detomidine, difenpiramide, difenpyramide, difisalamine, ditazol, emorfazone, fanetizole mesylate, fenflumizole, floctafenine, flumizole, flunixin, fluproquazone, fopirtoline, fosfosal, guaimesal, guaiazolene, isonixirn, lefetamine HCl, leflunomide, lofemizole, lotifazole, lysin clonixinate, meseclazone, nabumetone, nictindole, nimesulide, orgotein, orpanoxin, oxaceprolm, oxapadol, paranyline, perisoxal, perisoxal citrate, pifoxime, piproxen, pirazolac, pirfenidone, proquazone, proxazole, thielavin B, tiflamizole, timegadine, tolectin, tolpadol, tryptamid and those designated by company code number such as 480156S, AA861, AD1590, AFP802, AFP860, AI77B, AP504, AU8001, BPPC, BW540C, CHINOIN 127, CN100, EB382, EL508, F1044, FK-506, GV3658, ITF182, KCNTEI6090, KME4, LA2851, MR714, MR897, MY309, ONO3144, PR823, PV102, PV108, R830, RS2131, SCR152, SH440, SIR133, SPAS510, SQ27239, ST281, SY6001, TA60, TAI-901 (4-benzoyl-1-indancarboxylic acid), TVX2706, U60257, UR2301 and WY41770. Structurally related NSAIDs having similar analgesic and anti-inflammatory properties to the NSAIDs are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more corticosteroids, prodrug esters or pharmaceutically acceptable salts thereof for the treatment of TNF-mediated diseases, including acute and chronic inflammation such as rheumatic diseases, graft versus host disease and multiple sclerosis. Corticosteroids, prodrug esters and pharmaceutically acceptable salts thereof include hydrocortisone and compounds which are derived from hydrocortisone, such as 21-acetoxypregnenolone, alclomerasone, algestone, amcinonide, beclomethasone, betamethasone, betamethasone valerate, budesonide, chloroprednisone, clobetasol, clobetasol propionate, clobetasone, clobetasone butyrate, clocortolone, cloprednol, corticosterone, cortisone, cortivazol, deflazacon, desonide, desoximerasone, dexamethasone, diflorasone, diflucortolone, difluprednate, enoxolone, fluazacort, flucloronide, flumethasone, flumethasone pivalate, flunisolide, flucinolone acetonide, fluocinonide, fluorocinolone acetonide, fluocortin butyl, fluocortolone, fluorocortolone hexanoate, diflucortolone valerate, fluorometholone, fluperolone acetate, fluprednidene acetate, fluprednisolone, flurandenolide, formocortal, halcinonide, halometasone,

halopredone acetate, hydrocortamate, hydrocortisone, hydrocortisone acetate, hydrocortisone butyrate, hydrocortisone phosphate, hydrocortisone 21-sodium succinate, hydrocortisone tebutate, mazipredone, medrysone, meprednisone, methylprednicolone, mometasone furoate, paramethasone, prednicarbate, prednisolone, prednisolone 21-diedryaminoacetate, prednisolone sodium phosphate, prednisolone sodium succinate, prednisolone sodium 21-msulfobenzoate, prednisolone sodium 21-stearoglycolate, prednisolone tebutate, prednisolone 21-trimethylacetate, prednisone, prednival, prednylidene, prednylidene 21-diethylaminoacetate, tixocortol, triamcinolone, triamcinolone acetonide, triamcinolone benetonide and triamcinolone hexacetonide. Structurally related corticosteroids having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more slow-acting antirheumatic drugs (SAARDs) or disease modifying antirheumatic drugs (DMARDS), prodrug esters or pharmaceutically acceptable salts thereof for the treatment of TNF-mediated diseases, including acute and chronic inflammation such as rheumatic diseases, graft versus host and multiple sclerosis. SAARDs or DMARDS, prodrug esters and pharmaceutically acceptable salts thereof comprise: allocupreide sodium, auranofin, aurothioglucose, aurothioglycanide, azathioprine, brequinar sodium, bucillamine, calcium 3-aurothio-2-propanol-1-sulfonate, chlorambucil, chloroquine, clobuzarit, cuproxoline, cyclophosphamide, cyclosporin, dapsone, 15-deoxyspergualin, diacerein, glucosamine, gold salts (e.g., cycloquine gold salt, gold sodium thiomalate, gold sodium thiosulfate), hydroxychloroquine, hydroxyurea, kebuzone, levamisole, lobenzarit, melittin, 6-mercaptopurine, methotrexate, mizoribine, mycophenolate mofetil, myoral, nitrogen mustard, D-penicillamine, pyridinol imidazoles such as SKNF86002 and SB203580, rapamycin, thiols, thymopoietin and vincristine. Structurally related SAARDs or DMARDs having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more COX2 inhibitors, prodrug esters or pharmaceutically acceptable salts thereof for the treatment of TNF-mediated diseases, including acute and chronic inflammation. Examples of COX2 inhibitors, prodrug esters or pharmaceutically acceptable salts thereof include, for example, celecoxib. Structurally related COX2 inhibitors having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more antimicrobials, prodrug esters or pharmaceutically acceptable salts thereof for the treatment of TNF-mediated diseases, including acute and chronic inflammation. Antimicrobials include, for example, ampicillin, amoxycillin, aureomicin, bacitracin, ceftazidime, ceftriaxone, cefotaxime, cephachlor, cephalexin, cephradine, ciprofloxacin, clavulanic acid, cloxacillin, dicloxacillan, erythromycin, flucloxacillan, gentamicin, gramicidin, methicillan, neomycin, oxacillan, penicillin and vancomycin. Structurally related antimicrobials having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a more specific embodiment, the present invention is directed to the use of a TNFbp product(s) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more of the following compounds for the treatment of TNF-mediated diseases, including acute and chronic inflammation: granulocyte colony stimulating factor; thalidomide; BN 50730; tenidap; E 5531; tiapafant PCA 4248; nimesulide; panavir; rolipram; RP 73401; peptide T; MDL 201,449A; (1R,3S)-Cis-1-[9-(2,6-diaminopurinyl)]-3-hydroxy-4-cyclopentene hydrochloride; (1R,3R)-trans-1-[9-(2,6-diamino)purine]-3-acetoxycyclopentane; (1R,3R)-trans-1-[9-adenyl)-3-azidocyclopentane hydrochloride and (1R,3R)-trans-1-[6-hydroxy-purin-9-yl)-3-azidocyclopentane.

It is especially advantageous to formulate compositions of the additional anti-inflammatory compounds in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" as used herein refers to physically discrete units suited as unitary dosages for the patients to be treated, each unit containing a predetermined quantity of additional anti-inflammatory compounds calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coating, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like which are compatible with the active ingredient and with the mode of administration and other ingredients of the formulation and not deleterious to the recipient.

For oral therapeutic administration, the additional anti-inflammatory compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixers, suspensions, syrups, wafers and the like, or it may be incorporated directly with the food in the diet. The tablets, troches, pills, capsules and the like may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, alginic acid and the like; a lubricant such as magnesium stearate; a sweetening agent such as sucrose, lactose or saccharin; or a flavoring agent such as peppermint, oil of wintergreen or cherry or orange flavoring. When the dosage unit form is a capsule, it may contain, in addition to material of the type described herein, a liquid carrier. Various other materials may be present as a coating or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules may be coated with shellac, sugar or both. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the additional anti-inflammatory compound may be incorporated into a sustained-release preparation and formulation. The amount of the additional anti-inflammatory compound in such therapeutically useful composition is such that a suitable dosage will be obtained.

For parenteral therapeutic administration, each additional anti-inflammatory compound may be incorporated with a sterile injectable solution. The sterile injectable solution may be prepared by incorporating the additional anti-inflammatory compound in the required amount in an appropriate pharmaceutically acceptable carrier, with various other ingredients, followed by filtered sterilization. In the case of dispersions, each may be prepared by incorporating the additional anti-inflammatory compound into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated herein. In

the case of sterile injectable solutions, each may be prepared by incorporating a powder of the additional anti-inflammatory compound and, optionally, any additional desired ingredient from a previously sterile-filtered solution thereof, wherein the powder is prepared by any suitable technique (e.g., vacuum drying and freeze drying).

The use of such media and agents is well known in the art (see for example, *Remington's Pharmaceutical Sciences,* 18th Ed. (1990), Mack Publishing Co., Easton, Pa. 18042, pages 1435–1712, the disclosure of which is hereby incorporated by reference). Supplementary active ingredients can also be incorporated into the compositions.

The specific dose of the additional anti-inflammatory compound is calculated according to the approximate body weight or surface area of the patient. Other factors in determining the appropriate dosage can include the acute and chronic inflammatory disease or condition to be treated or prevented, the severity of the disease, the route of administration and the age, sex and medical condition of the patient. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the herein-mentioned formulations is routinely made by those skilled in the art. Dosages can also be determined through the use of known assays for determining dosages used in conjunction with appropriate dose-response data.

Thus, for example, it is within the scope of the invention that doses of the additional anti-inflammatory compounds selected for treating a particular acute or chronic inflammatory disease such as rheumatic diseases can be varied to achieve a desired therapeutic effect. Where one of the additional anti-inflammatory compounds has side effects, it can be given to patients during alternate treatment periods of combination therapy. For example, chronic methotrexate treatment is associated with gastrointestinal, hepatic, bone marrow and pulmonary toxicity (Sandoval et al. (1995), *British Journal of Rheumatology,* 34:49–56, the disclosure of which is hereby incorporated by reference).

Tests for monitoring the improvement of a disease can include specific tests directed, for example, to the determination of systemic response to inflammation, which include the erythrocyte sedimentation rate (ESR) and acute phase reactants (APR). Observations are made of the swelling, etc. of the affiicted body parts. Improvement in stiffness, and grip (where applicable), and reduction in pain of the patient is also observed. If the patient's condition is stable, the patient is re-treated at the same dosage weekly and is evaluated weekly. Provided the patient's condition is stable, the treatment may be continued. After six months of treatment, anatomical changes of the skeleton are determined by radiologic imaging, for example by X-radiography.

At the end of each period, the patient is again evaluated. Comparison of the pre-treatment and post-treatment radiological assessment, ESR and APR indicates the efficacy of the treatments. According to the efficacy of the treatments and the patient's condition, the dosage may be increased or maintained constant for the duration of treatment.

Preferably, the present invention is directed to a method with, optionally, one of the following combinations to treat or prevent TNF-mediated diseases, including acute and chronic inflammation such as rheumatic diseases and the symptoms associated therewith. One combination is a TNFbp product(s) (e.g., sTNFR-I, sTNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1) thereof) with one or more of methotrexate, leflunomide, an immunosuppressant (e.g., cyclosporin), ciprofloxacin, secreted or soluble fas antigen and an IL-1 inhibitor (e.g., IL-1ra). Preferred combinations include the TNFbp product(s) and methotrexate, or the TNFbp product(s) and leflunomide. Another combination is a TNFbp product(s) (e.g., STNFR-I, sTNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1) thereof) with one or more of methotrexate, leflunomide, sulphasazine and hydroxychloroquine.

In a specific preferred embodiment, the method comprises the administration (e.g., intra-articular, subcutaneous or intramuscular) of TNFbp product(s) (e.g., sTNFR-I, sTNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) in combination (pretreatment, post-treatment or concurrent treatment) with methotrexate and/or leflunomide and/or an IL-1 inhibitor (e.g., IL-1ra) and/or a secreted or soluble Fas antigen to treat rheumatic diseases.

In a specific preferred embodiment, the method comprises the administration (e.g., intravenous or intraventricular) of a TNFbp product(s) (e.g., sTNFR-I, sTNFR-II, STNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) in combination (pretreatment, post-treatment or concurrent treatment) with tissue plasminogen activator and/or an IL-1 inhibitor (e.g. IL-1ra) to treat brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous or intramuscular) of a TNFbp product(s) (e.g., sTNFR-I, sTNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) in combination (pretreatment, post-treatment or concurrent treatment) with one or more of a corticosteroid, cyclosporin, FK-506, or an interferon (e.g., alpha interferon, beta interferon, gamma interferon or consensus interferon) and/or an IL-1 inhibitor (e.g. IL-1ra, optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) to treat multiple sclerosis.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous or intramuscular) of a TNFbp product(s) (e.g., sTNFR-I, sTNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) in combination (pretreatment, post-treatment or concurrent treatment) with G-CSF and/or an IL-1 inhibitor (e.g. IL-1ra) to treat inflammatory bowel disease.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous or intramuscular) of a TNFbp product(s) (e.g., sTNFR-I, sTNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) in combination (pretreatment, post-treatment or concurrent treatment) with leptin, Marinol® or Megace® to treat cachexia/anorexia.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous or intramuscular) of a TNFbp product(s) (e.g., sTNFR-I, sTNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) in combination (pretreatment, post-treatment or concurrent treatment) with leptin to treat diabetes.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous, intraventricular or intrathecal) of a TNFbp product(s) (e.g., sTNFR-I, STNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) in combination (pretreatment, post-treatment or concurrent treatment) with an NSAID (e.g., indomethacin) and/or an IL-1 inhibitor (e.g. IL-1ra) to treat Alzheimer's disease.

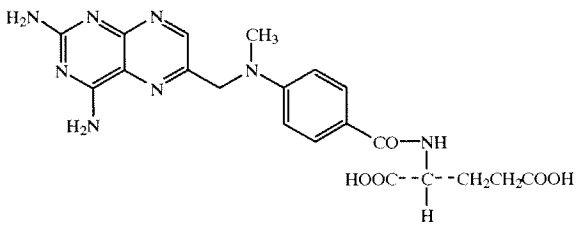
In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous, intraventricular or intrathecal) of a TNFbp product(s) (e.g., sTNFR-I, sTNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D siNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) optionally in combination (pretreatment, post-treatment or concurrent treatment) with a secreted or soluble fas antigen to treat cancer (e.g., leukemias); diabetes (e.g., juvenile onset Type 1 diabetes mellitus); graft versus host rejection; hepatitis; ischemic/reperfusion injury, including cerebral ischemia (brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration); neuroinflammatory diseases; rheumatic diseases, and tissue transplantation.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous, intraventricular or intrathecal) of a TNFbp product(s) (e.g., STNFR-I, STNFR-II, STNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) optionally in combination (pretreatment, post-treatment or concurrent treatment) with osteoprotogerin (European Patent Application No. 96309363.8) in the treatment of osteoporosis or Paget's disease.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous, intraventricular or intrathecal) of a TNFbp product(s) ((e.g., sTNFR-I, sTNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) in combination with gene therapy (e.g., using the human adenovirus) to modulate the inflammatory response to vector antigens (Zhang et al. (1997), *Arthritis & Rheumatism,* 40(9) :S220 (1138)).

The surprising and unexpected result disclosed herein is the ability of TNFbp product(s) (e.g., sTNFR-I, sTNFR-II, sTNFR fragments (2.6 D sTNFRs such as 2.6 D sTNFR-I) or sTNFR Fc(s) (sTNFR-I/IgG1 or sTNFR-II/IgG1), optionally formulated with a controlled release polymer (e.g., a dextran or hyaluronan)) and methotrexate to act synergistically in the treatment of various symptoms associated with TNF-mediated diseases, including acute and chronic inflammation such as rheumatic diseases. "Synergistically" is used herein to refer to a situation where the benefit conveyed by the joint administration of inhibitors is greater than the algebraic sum of the effects resulting from the separate administration of components of the combination. As shown in the experiments below, in the adjuvant arthritis model the combination treatment of TNFbp product(s) and methotrexate is synergistic with respect to treating systemic inflammation (i.e., splenomegaly) and weight loss associated with rheumatoid arthritis. Thus, the combined treatment with TNFbp product(s) and methotrexate has the advantage of achieving the same result with a lower dose or less frequent administration of methotrexate, thereby reducing any toxic effect and potentially the advantage of persisting even after the treatment has terminated.

Methotrexate is an anti-metabolite and immunosuppressive drug. Methotrexate is an effective anti-inflammatory agent with utility in the treatment of severe and disabling psoriasis and rheumatoid arthritis (Hoffmeister (1983), *The American Journal of Medicine,* 30:69–73 and Jaffe (1988), *Arthritis and Rheumatism,* 31:299). Methotrexate is N-[4-[(2,4-diamino-6-pteridinyl)methylamino]benzoyl]-L-glutamic acid and has the structural formula:



The following references describe the preparation of methotrexate (Seeger et al. (1949), *J. Am. Chem. Soc.,* 71:1753; the metabolism of methotrexate (Freeman (1958), *J. Pharmacol. Exp. Ther,* 122:154 and Henderson et al. (1965), *Cancer Res.,* 25:1008); the toxicity of methotrexate (Condit et al. (1960), *Cancer,* 13:222–249; the pharmacokinetic models of methotrexate (Bischoff et al. (1970), *J. Pharm, Sci.,* 59:149); the metabolism and pharmacokinetics of methotrexate (Evans (1980), *Appl. Pharmacokinet.,* Williams et al. (eds.), pp. 518–548 (Appl. Ther., Inc.); the clinical pharmacology of methotrexate (Bertino (1981), *Cancer Chemother.,* 3:359–375 and Jolivet et al. (1983), *N. Eng. J. Med.,* 309:1094–1104); and the clinical experience of methotrexate in rheumatoid arthritis (Weinblatt et al. (1985), *N. Eng. J. Med.,* 312:818–822; Furst (1985), *J. Rheumatol.,* 12(12) :1–14; Williams et al. (1985), *Arthritis Rheum.,* 28:721–730 and Seitz et al. (1995), *British Journal of Rheumatology,* 34:602–609). Additionally, numerous patents have been issued disclosing active agent methotrexate and methods for synthesizing methotrexate or potential intermediates in the synthesis of methotrexate: U.S. Pat. Nos. 2,512,572, 3,892,801, 3,989,703, 4,057,548, 4,067,867, 4,079,056, 4,080,325, 4,136,101, 4,224,446, 4,306,064, 4,374,987, 4,421,913 and 4,767,859.

The mechanism of action of methotrexate is poorly understood, however various activities of this drug have been demonstrated which likely contribute to its efficacy (Segal et al. (1990), *Seminars in Arthritis and Rheumatism,* 20:190–198). The following mechanisms of action for methotrexate have been postulated: inhibition of folate-dependent pathways and protein metabolism (Morgan et al. (1987), *Arthritis and Rheumatism,* 30:1348–1356); inhibition of neutrophil migration into arthritic joints (Van de Kerkhof et al. (1985), *British Journal of Dermatology,* 113:251–255; Ternowitz et al. (1987), *Journal of Investigative Dermatology,* 89:192–196 and Sperling (1992), *Arthritis and Rheumatism,* 35:376–384); IL-6 inhibitory activity (Segal (1991), *Arthritis and Rheumatism,* 34(2):146–152) and the local specific anti-proliferative effect on cells involved in arthritis (Rodenhuis et al. (1987), *Arthritis and Rheumatism,* 30:369–374). Methotrexate has been shown to block the interleukin-1 beta/interleukin-1 receptor pathway (Brody et al. (1993), *European Journal of Clinical Chemistry and Clinical Biochemistry,* 31(10):667–674); however, although methotrexate may inhibit the proliferative effects of IL-1 and decrease monocyte IL-1 production in the short

term in certain patients, this effect is not sustained and is unlikely to explain the long-term efficacy of methotrexate (Barrera et al. (1996), *Seminars in Arthritis and Rheumatism,* 25(4) :234–253).

Methotrexate may be administered orally, intraperitoneally, subcutaneously or intravenously. Oral administration is preferred. The following is an example of the procedure for the combined administration of a TNFbp product(s) and methotrexate to treat a human patient. The patient takes a tablet or capsule of methotrexate three times a week, at a total weekly dose of 5 to 50 mg/patient/week. The patient also is injected intravenously with TNFbp product(s), at a daily dose of 50 to 150 mg. It will be appreciated by those skilled in the art that the doses presented herein are the preferred doses. The starting dose of the particular compound(s) used is reduced for a patient who exhibits adverse reaction, or the drug used in combination with the compound(s) can be changed or reduced, e.g., depending on the different formulations, routes, dose schedules and/or other variables known to those skilled in the art, such as the individual patient's tolerance of the drug, its efficacy and toxicity.

Preferably, the patient is treated with a weekly starting dose of methotrexate at between 5 mg and 7.5 mg (orally or intramuscularly) and a daily dose of TNFbp product(s) at between 50 mg and 150 mg intravenously. The dosage of methotrexate is increased by 5 mg every 2 to 3 weeks. The maximum dosage level is determined at a point at which the patient shows improvements, which is generally preferably less than about 25 mg of methotrexate per week, more preferably between 5 to 25 mg of methotrexate per week. At the end of the five-day period the patient is evaluated. The evaluation includes physical examination and extensive laboratory testing. The tests include evaluation for toxicity. Additional laboratory monitoring in the case of methotrexate preferably includes a complete blood cell count every 2 weeks for the first 3 months and then monthly thereafter. Additional precautions preferably include monthly assessments of levels of serum albumin, alanine amino transferase, bilirubin, creatinine and blood urea nitrogen. Monthly urinalysis is also preferred.

The above is by way of example and does not preclude the treatment of other inflammatory joint diseases arising from abnormal or undesirably normal immune responses. The example also does not preclude other treatments to be used concurrently with these anti-inflammatory compounds that are known by those skilled in the art or that could be arrived at by those skilled in the art using the guidelines set forth in this specification. Other anti-inflammatory compounds mentioned above can be used in combination with the treatments.

EXAMPLES

Standard methods for many of the procedures described in the following examples, or suitable alternative procedures, are provided in widely recognized manuals of molecular biology such as, for example, Sambrook et al., *Molecular Cloning,* Second Edition, Cold Spring Harbor Laboratory Press (1987) and Ausabel et al., *Current Protocols in Molecular Biology,* Greene Publishing Associates/Wiley Interscience, New York (1990). All chemicals were either analytical grade or USP grade.

Example 1

An animal model of rheumatoid arthritis induced by an adjuvant was used to investigate the combination therapy of a TNF binding protein and methotrexate in male Lewis rats (3–7/group) weighing at least 200 g.

On day-0, all rats were injected with 100 μl of Freunds Complete Adjuvant (Sigma Chemical Co., St. Louis, Mo.) to which a synthetic adjuvant, N,N-dioctyldecyldecyl-N', N-bis(2-hydroxy-ethyl) propanediamine, 50 mg/ml, was added. On days 0–14 methotrexate in 1% carboxymethylcellulose (Sigma) was orally administered daily (0.06 mg/kg) to two groups of rats. On days 8, 10, 12, and 14, *E. coli*-derived c105 sTNFR-I dimerized with PEG-20,000-bis-vinyl sulfone (c105 sTNFR-I dumbbell; prepared generally in accordance with the teachings of WO 95/34326) formulated in pharmaceutical composition (34 mM NaCl, 10 mM sodium phosphate, 4% sorbitol (w/v) in water; pH 6.5) was administered by subcutaneous (SC) injection (3 mg/kg) to one group of rats being treated with both Freunds Complete Adjuvant and methotrexate and to another group of rats being treated with Freunds Complete Adjuvant alone.

Body weights were taken on day 0 and every other day from day 9 to termination on day 15. Caliper measurements and clinical scoring were done on day 9 and every other day until termination. At this time animal's body, paw and spleen weights were determined.

As seen in FIGS. **3** and **4**, rats treated with c105 sTNFR-I dumbbell alone exhibited about 42% inhibition of paw swelling (area under the curve—AUC), no significant benefit on splenomegaly (not shown) and about 13.2% inhibition of body weight change (not shown). Rats treated with methotrexate had 26% inhibition of paw swelling (AUC), no inhibition of spleen weight (not shown) and 3% inhibition of body weight change (not shown). The combination therapy provided 75% inhibition of paw swelling (AUC), 48% inhibition of splenomegaly (not shown) and 16.2% inhibition of body weight change (not shown).

As seen in FIG. **5**, the final analysis (inhibition at termination) of terminal paw weights and spleen weights indicated that c105 sTNFR-I dumbbell alone resulted in 10.9% inhibition of paw inflammation, 30.4% inhibition of splenomegaly and 13.2% inhibition of body weight change (not shown). Methotrexate treatment alone gave only a 3.9% inhibition of paw inflammation, 8.5% inhibition of splenomegaly and 3% inhibition of body weight change (not shown). The combination of c105 sTNFR-I dumbbell and methotrexate resulted in a 46.8% inhibition of paw swelling, 48% inhibition of splenomegaly and 16.2% inhibition of body weight change (not shown).

Example 2

An animal model of rheumatoid arthritis induced by an adjuvant was used to investigate the combination therapy of a TNF binding protein and methotrexate in male Lewis rats (5–7/group) weighing at least 200 g.

On day-0, all rats were injected with 100 μl of Freunds Complete Adjuvant (Sigma Chemical Co., St. Louis, Mo.) to which a synthetic adjuvant, N,N-dioctyldecyldecyl-N', N-bis(2-hydroxy-ethyl) propanediamine, 50 mg/ml, was added. On day 0–14 methotrexate in 1% carboxymethylcellulose (Sigma) was orally administered daily (0.06 mg/kg) to two groups of rats. On days 9, 11, and 13, CHO-derived sTNFR-II/hIgG1 fusion protein (sTNFR-II Fc; prepared generally in accordance with the teachings of EP 418 014) formulated in pharmaceutical composition (34 mM NaCl, 10 mM sodium phosphate, 4% sorbitol (w/v) in water; pH 6.5) was administered by subcutaneous infusion (18 mg/kg) to one group of rats being treated with both Freunds Complete Adjuvant and methotrexate and to another group of rats being treated with Freunds Complete Adjuvant alone.

Body weights were taken on day 0 and every other day from day 9 to termination on day 15. Caliper measurements and clinical scoring were done daily from day 9 until termination on day 15. At this time animal's body, paw and spleen weights were determined.

As seen in FIG. **6**, rats treated with sTNFR-II Fc alone exhibited about 8% inhibition of paw swelling (area under the curve—AUC), with no significant benefit on splenomegaly (−7%) or body weight change (−5%). Rats treated with methotrexate had 66% inhibition of paw swelling (AUC), 74% inhibition of spleen weight and 64% inhibition of body weight change. The combination therapy provided 96% inhibition of paw swelling (AUC), 94% inhibition of splenomegaly and 79% inhibition of body weight change.

As seen in FIG. **7**, the final analysis (inhibition at termination) of terminal paw weights indicated that sTNFR-II Fc alone resulted in 10% inhibition of paw inflammation, methotrexate treatment alone gave a 74% inhibition of paw inflammation and the combination of sTNFR-II Fc and methotrexate resulted in a 88% inhibition of paw swelling.

Example 3

The combination immunotherapeutic effects of c105 sTNFR-I dumbbell and fas fusion protein were assessed using a mouse model of D-Galactosamine (D-$GalNH_2$) induced lethality. The D-galactosamine (D-$GalNH_2$)/Lipopolysaccharide (LPS) model (Mountz et al., *J. Immunology,* 155:4829–4837). In this model, MRL-lpr/lpr autoimmune mice are administered D-$GalNH_2$with bacterial endotoxin (LPS), and lethality is observed through +96 hours post challenge.

Materials and Methods

Dihydrofolate reductase (DHFR) deficient Chinese hamster ovary cells (CHOd-cells) were transfected with fas/hIgG1 chimeric cDNA (Mountz, et al. (1996), "Autoimmunity Due to Defective Nur-77, Fas and TNF-R1 Apoptosis" in Mechanisms of Lymphocyte Activation and Immune Regulation, Vol. 6, p241–262 (Gupta and Cohen (Eds)), Plenem Press, NY) in pDSRα2, generally in accordance with the disclosure of DeClerck, et al. (1991), *JCB,* 266:3893–3899. The transfection procedure differed from the protocol of set forth in DeClerck, et al. (1991), supra, as follows: the cells were transfected with 800,000 cells, with 10 micrograms and 8 micrograms of herring sperm as a carrier, and the cells were split at 2 days post-transfection.

Following expression of the fas fusion protein, the protein was purified using a Protein G Sepharose Fast Flow, generally in accordance with Jungbauer, et al. (1989), *J. Chrom.,* 476:257–268. The purified protein was formulated in Phosphate buffered saline (Gibco BRL, Grand Island, N.Y.).

Protocol

After overnight fasting, 6–8 week old female MRL-lpr/lpr mice (Jackson Laboratory, Bar Harbor, Me. (5/7/group) were cannulated with jugular catheters and allowed to recover for several days. They were then placed in infusion cages and acclimated for a week prior initiating saline infusion.

At hour-0, all mice were injected intraperitoneally with 31 micrograms of D-$GalNH_2$ (Sigma) suspended in Hank's Balanced Salt Solution (Gibco BRL) (120 micrograms/ml); and lipopolysaccharide (LPS) from *E. coli* Serotype 0127:B8 (Sigma) in sterile, endotoxin-free phosphate buffered saline (PBS) (6 micrograms/mouse).

At 0-hour–+2 hours post-challenge, fas fusion protein formulated in a pharmaceutical composition (Phosphate buffered saline (Gibco BRL, Grand Island, N.Y.)) was administered intravenously in serial 2-fold dilutions (microgram/kg dosages) to two groups of mice.

At 0-hour–+2 hours post-challenge, c105 sTNFR-I dumbbell formulated in a pharmaceutical composition (34 mM NaCl, 10 mM sodium phosphate, 4% sorbitol (w/v) in water; pH 6.5) was administered intravenously in serial 2-fold dilutions (microgram/kg dosages) to one group of mice being treated with both D $GalNH_2$ and fas fusion protein and to another group of mice being treated with D-$GalNH_2$ alone.

ED_{50} curves were generated with statistical software for the MacIntosh (Statview®, Mountain View, Calif.). Lethality was followed through +96 hour after challenge.

Results

As seen in FIG. **8**, mice administered c105 sTNFR-I dumbbell (100 micrograms/kg; N=6; I.V.) at time=−1 hour before challenge were observed to be completely protected (100% survival) against LPS challenge in comparison to control (saline-treated) mice (N=6) challenged with LPS/D-$GalNH_2$ ($P<0.01$). Mice treated with sub-optimal doses of c105 sTNFR-I dumbbell (25 micrograms/kg; N=6) were observed to have ~35% protection through +96 hours after challenge. All mice treated with fas fusion protein (100 micrograms/kg; N=6) were dead by +24 hours-post challenge. However, when mice (N=6) were treated I.V. with both c105 sTNFR-I dumbbell (25 micrograms/kg) and fas fusion protein (100 micrograms /kg), enhanced survival (70%) was observed through +36 hours in comparison to either the c105 sTNFR-I dumbbell treated (25 micrograms), fas fusion protein (100 micrograms/kg), or disease control animals alone ($P<0.05$). These results suggest that c105 sTNFR-I dumbbell and fas fusion protein are synergistic in their therapeutic effects in the LPS/D-$GalNH_2$ model of acute inflammation.

The foregoing description of the invention is exemplary for purposes of illustration and explanation. It will be apparent to those skilled in the art that changes and modifications are possible without departing from the spirit and scope of the invention. It is intended that the following claims be interpreted to embrace all such changes and modifications.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 4

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 483 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..483

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG       48
Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser
  1               5                  10                  15

ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT       96
Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys
             20                  25                  30

CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC      144
Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser
         35                  40                  45

TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA      192
Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys
     50                  55                  60

TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC      240
Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
 65                  70                  75                  80

CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG      288
Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp
                 85                  90                  95

AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC AAT GGG      336
Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly
            100                 105                 110

ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC      384
Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys
        115                 120                 125

CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC      432
His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys Ser Asn
    130                 135                 140

TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT GAG      480
Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu
145                 150                 155                 160

AAT                                                                  483
Asn
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 161 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser
  1               5                  10                  15

Ile Cys Cys Thr Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys
             20                  25                  30

Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser
         35                  40                  45

Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys
     50                  55                  60
```

-continued

```
Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
 65                  70                  75                  80

Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp
                85                  90                  95

Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu Asn Gly
            100                 105                 110

Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys
        115                 120                 125

His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val Ser Cys Ser Asn
    130                 135                 140

Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu
145                 150                 155                 160

Asn
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 705 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..705

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TTG CCC GCC CAG GTG GCA TTT ACA CCC TAC GCC CCG GAG CCC GGG AGC       48
Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr Ala Pro Glu Pro Gly Ser
  1               5                  10                  15

ACA TGC CGG CTC AGA GAA TAC TAT GAC CAG ACA GCT CAG ATG TGC TGC       96
Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln Thr Ala Gln Met Cys Cys
            20                  25                  30

AGC AAG TGC TCG CCG GGC CAA CAT GCA AAA GTC TTC TGT ACC AAG ACC      144
Ser Lys Cys Ser Pro Gly Gln His Ala Lys Val Phe Cys Thr Lys Thr
        35                  40                  45

TCG GAC ACC GTG TGT GAC TCC TGT GAG GAC AGC ACA TAC ACC CAG CTC      192
Ser Asp Thr Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu
    50                  55                  60

TGG AAC TGG GTT CCC GAG TGC TTG AGC TGT GGC TCC CGC TGT AGC TCT      240
Trp Asn Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser
65                  70                  75                  80

GAC CAG GTG GAA ACT CAA GCC TGC ACT CGG GAA CAG AAC CGC ATC TGC      288
Asp Gln Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys
                85                  90                  95

ACC TGC AGG CCC GGC TGG TAC TGC GCG CTG AGC AAG CAG GAG GGG TGC      336
Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys
            100                 105                 110

CGG CTG TGC GCG CCG CTG CGC AAG TGC CGC CCG GGC TTC GGC GTG GCC      384
Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg Pro Gly Phe Gly Val Ala
        115                 120                 125

AGA CCA GGA ACT GAA ACA TCA GAC GTG GTG TGC AAG CCC TGT GCC CCG      432
Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro
    130                 135                 140

GGG ACG TTC TCC AAC ACG ACT TCA TCC ACG GAT ATT TGC AGG CCC CAC      480
Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg Pro His
145                 150                 155                 160

CAG ATC TGT AAC GTG GTG GCC ATC CCT GGG AAT GCA AGC AGG GAT GCA      528
```

-continued

```
Gln Ile Cys Asn Val Val Ala Ile Pro Gly Asn Ala Ser Arg Asp Ala
                165                 170                 175

GTC TGC ACG TCC ACG TCC CCC ACC CGG AGT ATG GCC CCA GGG GCA GTA      576
Val Cys Thr Ser Thr Ser Pro Thr Arg Ser Met Ala Pro Gly Ala Val
            180                 185                 190

CAC TTA CCC CAG CCA GTG TCC ACA CGA TCC CAA CAC ACG CAG CCA ACT      624
His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln His Thr Gln Pro Thr
        195                 200                 205

CCA GAA CCC AGC ACT GCT CCA AGC ACC TCC TTC CTG CTC CCA ATG GGC      672
Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser Phe Leu Leu Pro Met Gly
    210                 215                 220

CCC AGC CCC CCA GCT GAA GGG AGC ACT GGC GAC                          705
Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp
225                 230                 235


(2) INFORMATION FOR SEQ ID NO:4:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 235 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr Ala Pro Glu Pro Gly Ser
  1               5                  10                  15

Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln Thr Ala Gln Met Cys Cys
             20                  25                  30

Ser Lys Cys Ser Pro Gly Gln His Ala Lys Val Phe Cys Thr Lys Thr
         35                  40                  45

Ser Asp Thr Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu
     50                  55                  60

Trp Asn Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser
 65                  70                  75                  80

Asp Gln Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys
                 85                  90                  95

Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys
            100                 105                 110

Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg Pro Gly Phe Gly Val Ala
        115                 120                 125

Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro
    130                 135                 140

Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg Pro His
145                 150                 155                 160

Gln Ile Cys Asn Val Val Ala Ile Pro Gly Asn Ala Ser Arg Asp Ala
                165                 170                 175

Val Cys Thr Ser Thr Ser Pro Thr Arg Ser Met Ala Pro Gly Ala Val
            180                 185                 190

His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln His Thr Gln Pro Thr
        195                 200                 205

Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser Phe Leu Leu Pro Met Gly
    210                 215                 220

Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp
225                 230                 235
```

We claim:

1. A pharmaceutical composition comprising a TNF binding protein and a Fas antigen.

2. The pharmaceutical composition of claim 1, wherein the Fas antigen is a fas fusion protein.

3. The pharmaceutical composition of claim 1, wherein said TNF binding protein is sTNFR-I, sTNFR-II, sTNFR fragments or sTNFR Fc.

4. The pharmaceutical composition of claim 1, wherein said TNF binding protein is present in an amount of up to about 20 mg.

5. A method for treating septic shock which comprises administering to a patient in need thereof therapeutically effective amounts of a TNF binding protein which is administered prior to, concurrently with or after administration of a Fas antigen.

6. The method of claim 5, wherein the Fas antigen is a fas fusion protein.

7. The method of claim 6, wherein said TNF binding protein and said fas fusion protein are administered in a pharmaceutically acceptable carrier.

8. The method of claim 5, wherein said TNF binding protein is sTNFR-I, sTNFR-II, sTNFR fragments or sTNFR Fc.

* * * * *

Int. J. Cancer: **48,** 744–748 (1991)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

A NOVEL RECOMBINANT TUMOR NECROSIS FACTOR-ALPHA MUTANT WITH INCREASED ANTI-TUMOR ACTIVITY AND LOWER TOXICITY

Satoshi NAKAMURA[1,3], Arata KATO[1], Tsukio MASEGI[1], Masami FUKUOKA[1], Kazuo KITAI[1], Hiroko OGAWA[1], Yataro ICHIKAWA[1], Masahiro MAEDA[2], Naoki WATANABE[2], Yutaka KOHGO[2] and Yoshiro NIITSU[2]

[1]*Biotechnology Research Laboratories, Teijin Limited, Hino-shi, Tokyo 191; and* [2]*Department of Internal Medicine (Section 4), Sapporo Medical College, Sapporo-shi, Hokkaido 060, Japan.*

We prepared a novel recombinant tumor necrosis factor-α (TNF) mutant (mutant 471), in which 7 N-terminal amino-acids were deleted and Pro[8]Ser[9]Asp[10] was replaced by ArgLysArg, and compared its biological activity with that of wild-type recombinant TNF. Mutant 471 had a 7-fold higher anti-tumor activity against murine L-M cells *in vitro*, and a higher binding activity to TNF receptors on L-M cells, than wild-type TNF. Furthermore, mutant 471 showed a higher anti-tumor effect on murine Meth A-HM tumors transplanted into BALB/c mice, with complete regression of the tumors being observed in the animals. The possible cachectin activity of mutant 471 was almost the same as that of wild-type TNF. The acute lethal toxicity of mutant 471 in β-D-galactosamine-sensitized C3H/HeJ mice was 18 times lower than that of wild-type TNF. These results suggest that mutant 471 might be a more promising anti-cancer agent than wild-type TNF.

Tumor necrosis factor-α (TNF) was originally identified by Carswell *et al.* (1975) in the serum of mice infected with Bacillus Calmette Guérin and subsequently treated with endotoxin. TNF induces the hemorrhagic necrosis of transplanted tumors *in vivo*, and is cytotoxic towards various tumor cell lines *in vitro* (Old, 1985). However, the mechanism of its anti-tumor effects remains largely unexplained, although some studies have suggested the involvement of hydroxyl radical production (Yamauchi *et al.*, 1989, 1990) and of damage to tumor vessels (Watanabe *et al.*, 1988*a*).

The gene for human TNF was cloned and expressed in *Escherichia coli* (Pennica *et al.*, 1984; Shirai *et al.*, 1985; Nakamura *et al.*, 1990), making large quantities of pure TNF available for clinical trials in cancer patients. Unfortunately, recombinant TNF has failed to produce a satisfactory anti-cancer effect when administered alone, particularly when used for systemic therapy (Blick *et al.*, 1987; Creaven *et al.*, 1987; Kimura *et al.*, 1987). This appears to be largely because recombinant TNF is highly toxic and therefore adequate doses cannot be safely administered. To overcome this disadvantage, attempts have been made to combine TNF with γ-interferon (Talmadge *et al.*, 1987; Watanabe *et al.*, 1988*c*), various anti-cancer agents (Regenass *et al.*, 1987), and hyperthermia (Watanabe *et al.*, 1988*b*; Niitsu *et al.*, 1988*a*).

Several TNF mutants with some advantages over the wild-type recombinant TNF have been prepared by protein engineering techniques. TNF mutants with deletions at the N-terminal (Creasey *et al.*, 1987; Nakamura *et al.*, 1991) and a mutant with the substitution of Leu[157] by Phe (Kamijo *et al.*, 1989) have a higher anti-tumor activity *in vitro*. Also, TNF mutants with the N-terminal amino-acids substituted by basic amino acids (Soma *et al.*, 1987) have a broader anti-tumor spectrum. However, most of these TNF mutants have only been studied *in vitro* so far.

In this study, we prepared a TNF mutant (mutant 471) by deleting 7 amino-acids at the N-terminal and replacing Pro[8]Ser[9]Asp[10] by ArgLysArg. Mutant 471 had an increased anti-tumor activity both *in vitro* and *in vivo*, as well as a higher binding activity to TNF receptors, when compared with wild-type TNF. The level of the cachectin activity of mutant 471 was almost the same as that of wild-type TNF. Mutant 471 exhibited a lower acute lethal toxicity in β-D-galactosamine-sensitized mice.

MATERIAL AND METHODS

Preparation of recombinant TNFs

The expression plasmid pTNF471 encoding mutant 471 was derived from the plasmid pTNF401A (Nakamura *et al.*, 1990) coding for wild-type TNF by cassette mutagenesis. Details of the construction of pTNF471 will be published elsewhere.

The *E. coli* strain C600r$^-$m$^-$ (ATCC 33525) transformed by the plasmids pTNF471 or pTNF401A was cultured as described by Nakamura *et al.* (1988). Cells were harvested, sonicated and centrifuged to obtain bacterial lysates. To remove endotoxin and nucleic acids from the lysates, β-(1,4)-2-amino-2-deoxy-D-glucan (C-9) (Kurita Water Industries, Tokyo) was added to the lysates and further centrifugation was performed. The supernatants were passed through an Affi-Gel 10 affinity column (Bio-Rad, Richmond, CA) coupled with an anti-TNF monoclonal antibody (Yone *et al.*, 1987). Mutant 471 or wild-type TNF was eluted from the column with 100 mM citrate buffer (pH 3.0). The purity of each type of TNF was more than 99%, as determined by SDS-PAGE (Laemmli, 1970). The endotoxin content of each sample was less than 20 pg/mg-protein, as determined by the *Limulus* amebocyte lysate assay with commercial reagents (Seikagaku Kogyo, Tokyo).

Cytotoxic assays

In vitro anti-tumor activity was determined by the method of Yamauchi *et al.* (1989). One unit (U) was defined as the quantity of TNF required to kill 50% of 1.0×10^4 L-M cells (murine tumorigenic fibroblasts) (ATCC CCL 1.2) during a 24-hr incubation.

The *in vivo* assay was performed according to the procedure of Carswell *et al.* (1975). Meth A-HM (Niitsu *et al.*, 1988*b*), a highly TNF-sensitive subline derived from Meth A murine fibrosarcoma, was used as the target. Male BALB/c mice (Charles River, Kanagawa) with an average body weight of 20 g, were used at 8 weeks of age. Tumor weights were estimated from linear measurements using the formula $a_T^2 \times b_T/2 - a_N^2 \times b_N/2$, where a_T represents the shortest diameter in mm, b_T the longest diameter in mm, a_N the shortest diameter of tumor necrosis, and b_N the longest diameter of tumor necrosis. The degree of hemorrhagic necrosis in the tumor (necrotic response) was scored as described by Carswell *et al.* (1975).

[3]To whom correspondence and requests for reprints should be sent, at the Department of Bioengineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259, Nagatsuta, Midori-ku, Yokohama 227, Japan.

Received: January 10, 1991 and in revised form April 2, 1991.

Receptor binding assay

Mutant 471 and wild-type TNF were radioiodinated with Iodogen (Pierce, Rockford, IL) (Fraker and Speck, 1978). The specific activities of the ^{125}I-labelled TNFs so obtained were about 6.5×10^5 cpm/μg and these ^{125}I-labelled molecules retained full biological activities (data not shown).

The binding assay for TNF receptors on L-M cells was carried out as previously described (Niitsu *et al.*, 1988*a,b*; Watanabe *et al.*, 1988*c*). The number of TNF receptors per cell and the dissociation constant (K_D) were determined by Scatchard plot analysis.

Lipoprotein lipase assay

Lipoprotein lipase (LPL) activity in 3T3-L1 cells (murine preadipocytes) (ATCC CL 173) was assayed by the method of Kawakami *et al.* (1982, 1987).

Acute lethal toxicity

Female C3H/HeJ mice were obtained from Clea Japan (Tokyo). Eight-week-old mice with an average body weight of 17 g were used for the lethality tests. Several doses of mutant 471 or wild-type TNF were administered intraperitoneally (i.p.) in 500 μl of saline containing 18 mg of β-D-galactosamine. Lethality was checked 24 hr after the administration of TNF, and lethal toxicity was expressed as the 50% lethal dose (LD_{50}).

RESULTS

In vitro anti-tumor activity

We compared the anti-tumor activity of purified mutant 471 and wild-type TNF for L-M cells *in vitro*. As shown in Figure 1, mutant 471 killed L-M cells more effectively than wild-type TNF, the specific activities of the mutant 471 and wild-type TNF being 2.00×10^7 U/mg and 3.03×10^6 U/mg, respectively.

Receptor binding activity

The results of the binding assay for TNF receptors are summarized in Table I. The numbers of TNF receptors on the surface of L-M cells for these 2 types of TNF were about 5,000 per cell and no differences was observed, suggesting that mutant 471 and wild-type TNF would bind to the same TNF receptors. The K_D values of mutant 471 and wild-type TNF were 4.08×10^{-9} M and 1.65×10^{-9} M, respectively. Thus, the binding activity to TNF receptor of mutant 471 was higher than that of wild-type TNF.

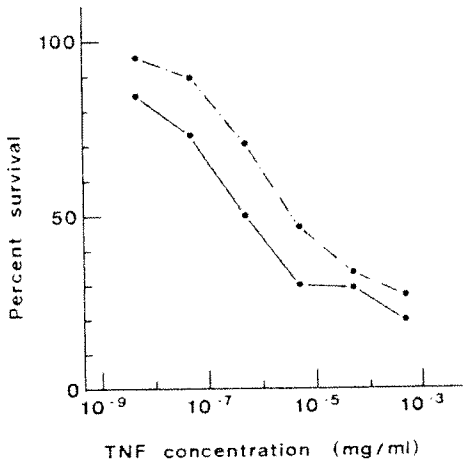


FIGURE 1 – *In vitro* cytotoxic activity of mutant 471 and wild-type TNF for L-M cells. L-M cells were seeded into 96-well microplates at 1.0×10^4 cells/well, incubated at 37°C for 4 hr, and then various concentrations of mutant 471 or wild-type TNF solutions were added to the wells. After incubation for 24 hr, the surviving cells were counted. Values shown are the means of triplicate determinations. The percent survivals (ordinate) were plotted against the concentrations of each form of TNF (abscissa). —•—•—, wild-type TNF; ———, mutant 471.

In vivo *anti-tumor activity*

The anti-tumor activity of mutant 471 and wild-type TNF was compared using Meth A-HM cells transplanted into BALB/c mice (5×10^5 cells intradermally (i.d.) implanted into the lateral abdominal wall). When the tumors had reached 6–8 mm in diameter (day zero), 50 μg of mutant 471 or wild-type TNF in phosphate-buffered saline (PBS) were administered intravenously (i.v.). Subsequently, the same dose of each TNF was administered on days 2, 6, 8 and 10. Control mice received PBS according to the same schedule as that for the experimental groups. Figure 2 shows the changes in relative tumor weight after the first administration of each type of TNF. Mutant 471 caused more tumor growth suppression than wild-type TNF at the same dose. The average necrotic response caused by mutant 471 was + + +, whereas it was + + for wild-type TNF.

A significant prolongation of survival compared to control mice was seen in the mice treated with mutant 471, but little prolongation was seen in the wild-type TNF group (Fig. 3). Complete regression of the tumors was observed on day 15 in 2 of the 5 mice in the mutant 471 group.

Effect of TNF on LPL activity in vitro

Cachectin, which was presumed to be responsible for cachexia in cancer patients, has been purified as a fraction containing LPL suppression activity and reported to be identical to TNF (Beutler *et al.*, 1985; Beutler and Cerami, 1986). Therefore, we compared the cachectin activity of mutant 471 and wild-type TNF by monitoring the suppression of LPL activity *in vitro*. As shown in Table II, the suppression of LPL activity by mutant 471 only occurred to the same degree as that induced by wild-type TNF.

Acute lethal toxicity

We examined the acute lethal toxicity of mutant 471 and wild-type TNF in β-D-galactosamine-sensitized C3H/HeJ mice. Representative results for each type of TNF at varying doses are shown in Table III. The mice showed a 100% death rate after i.p. administration of 16 μg/kg of wild-type TNF, but only a 20% death rate at the same dose of mutant 471. The LD_{50} values for mutant 471 and wild-type TNF were 35 μg/kg and 2 μg/kg, respectively. Thus, mutant 471 had about 18 times as low lethal toxicity as did wild-type TNF in β-D-galactosamine-sensitized mice.

DISCUSSION

We compared the anti-tumor activity of mutant 471 with that of wild-type TNF using the standard assays for TNF: an *in vitro*

TABLE I – NUMBERS OF TNF RECEPTORS ON L-M CELLS AND K_D VALUES FOR WILD-TYPE TNF AND MUTANT 471

TNF	Receptors/cell	K_D (M)
Wild-type TNF	5.04×10^3	1.65×10^{-9}
Mutant 471	5.06×10^3	4.08×10^{-9}

L-M cells (5.0×10^5 cells) were incubated with ^{125}I-labelled TNFs for 1 hr at 4°C. The cells were harvested and the specific bindings of TNFs were determined by gamma counting. The numbers of TNF receptors per cell and K_D values were determined by Scatchard plot analysis.

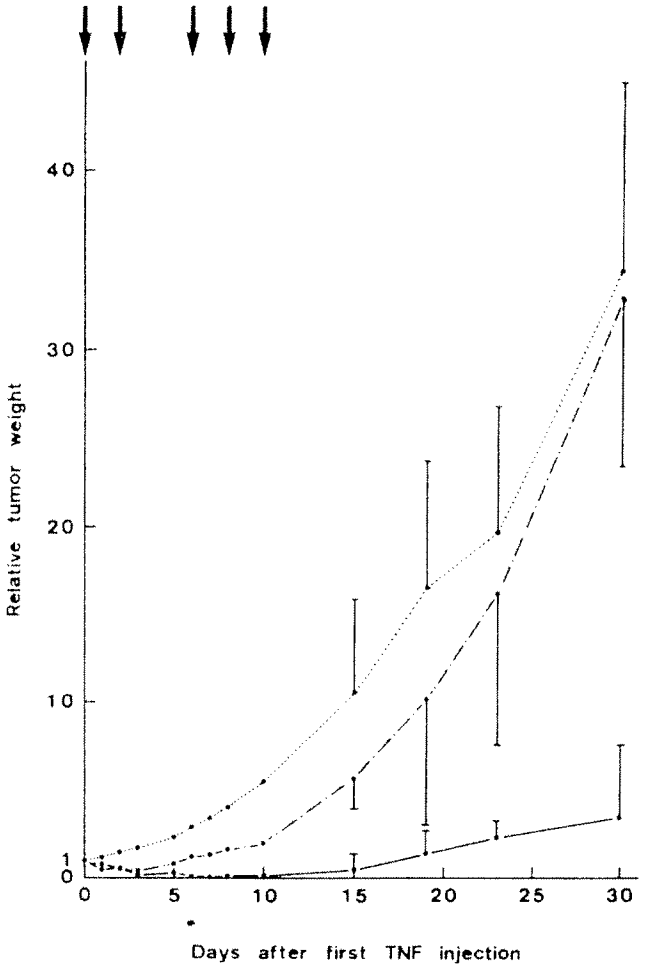


FIGURE 2 – Changes in the relative tumor weights in wild-type TNF-treated, mutant 471-treated, and control BALB/c mice with i.d. transplanted Meth A-HM fibrosarcomas. Fifty micrograms of TNF were injected i.v. on days 0, 2, 6, 8 and 10 (↓). •••, control; —•—•—, wild-type TNF; ———, mutant 471. Values are the mean ± SD (bars) for groups of 5 mice. $p < 0.005$ (mutant 471 group *vs.* control group); $p < 0.05$ (mutant 471 group *vs.* wild-type TNF group).

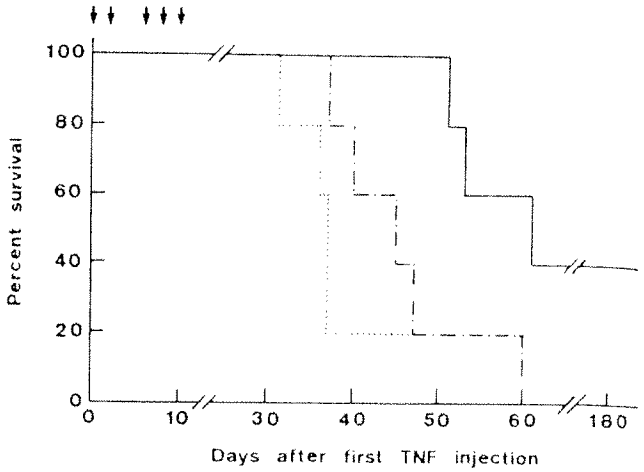


FIGURE 3 – Survival of BALB/c mice (5 mice per group) bearing Meth A-HM fibrosarcoma. The treatment regimen was the same as in Figure 2. •••, control; —•—•—, wild-type TNF; ———, mutant 471.

TABLE II – EFFECT OF WILD-TYPE TNF OR MUTANT 471 ON LPL ACTIVITY IN 3T3-L1 CELLS

TNF	TNF concentration (μg/ml)	LPL activity (mU/ml)[%]
—	0	5.38 ± 0.04 [100.0]
Wild-type TNF	2.0×10^{-4}	2.39 ± 0.14 [44.5]
Mutant 471	2.0×10^{-4}	2.65 ± 0.22 [49.3]

Wild-type TNF or mutant 471 was added to 3T3-L1 cultures and the cells were incubated at 37°C for 18 hr. After incubation, the LPL activity released by heparin into the medium was assayed. The data of LPL activity represent the mean ± SD (n = 4). One unit of LPL activity was defined as described by Kawakami *et al.* (1982, 1987).

L-M killing assay and the *in vivo* determination of growth inhibition and necrotic response for a Meth A sarcoma subline transplanted into BALB/c mice. Mutant 471 had a 7-fold higher *in vitro* anti-tumor activity against L-M cells than wild-type TNF. It showed an increase in tumor-cell receptor-binding activity, so the greater direct cytotoxicity of mutant 471 was probably due to its higher receptor-binding activity. Mutant 471 also showed a higher anti-tumor effect *in vivo*, and complete regression of the tumor was observed in some mice. North and others (Havell *et al.*, 1988; North and Havell, 1988) have indicated that *in vivo* necrosis-inducing activity is responsible for destruction of the tumor vasculature and does not depend on *in vitro* anti-tumor activity. It appears likely, therefore, that endothelial cells have a higher affinity for mutant 471 than for wild-type TNF.

On the other hand, mutant 471 had a lower acute lethal toxicity in β-D-galactosamine-sensitized normal C3H/HeJ mice than did wild-type TNF. Only normal cells take part in lethal toxicity in normal mice, although what kind of normal cells are involved in lethal toxicity is unclear. Thus, the difference in the response to mutant 471 between normal cells and tumor cells can be accounted for by the differences in the species of related receptor molecules or the pathways of signal transduction. The possible cachectin activity of mutant 471 was the same as that of wild-type TNF, so our findings suggest that mutant 471 might be more effective and less toxic than wild-type TNF.

Gatanaga *et al.* (1989) have also reported novel recombinant TNF mutants (TNF-S_{AM1} and TNF-S_{AM2}) with a higher anti-tumor activity and a lower toxicity than wild-type TNF. However, the relative *in vitro* anti-tumor activity of mutant 471 to wild-type TNF was higher than that of the TNF-S series mutants. The acute lethal toxicity of the TNF-S series in normal BALB/c mice and Meth A tumor-bearing BALB/c mice was, respectively, one-third and one-fourth of that of wild-type TNF (LD_{50} value in μg/kg body weight). The acute lethal toxicity of mutant 471 in β-D-galactosamine-sensitized C3H/HeJ mice was 18 times lower than that of wild-type TNF. We cannot directly compare the toxicity of TNF in β-D-galactosamine-sensitized mice with its toxicity in normal or tumor-bearing mice. However, the LD_{50} values of the TNF-S series for Meth A tumor-bearing BALB-c mice were less than 1 mg/kg, while mutant 471 was safely administered at a dose of more than 2 mg/kg. Thus, a superior toxicity profile can be expected with mutant 471. Also, the maximum complete regression rate for mutant 471 (2 of 5 mice) was higher than that for the TNF-S series mutants (1 of 4 mice).

At present, the relative *in vitro* anti-tumor activity of mutant 471 in comparison with that of wild-type TNF was the highest of all the TNF mutants reported so far. Furthermore, mutant

TABLE III – ACUTE LETHAL TOXICITY OF WILD-TYPE TNF AND MUTANT 471 IN β-D-GALACTOSAMINE-SENSITIZED C3H/HeJ MICE

TNF	Lethality (dead mice/total number of mice)						LD_{50} (μg/kg)
	Dose (μg/kg)						
	1	4	16	64	128	356	
Wild-type TNF	0/5	5/5	5/5	5/5	5/5	5/5	2
Mutant 471	0/5	0/5	1/5	4/5	4/5	5/5	35

Each form of TNF at various doses and 18 mg of β-D-galactosamine in 500 μl of saline were injected i.p. into female C3H/HeJ mice with an average body weight of 17 g. Survival/death was observed 24 hr later.

471 might be superior in anti-tumor effect *in vivo* and with less toxicity compared to TNF-S series, the only TNF mutants which had been studied *in vivo*. However, more detailed studies are necessary before clinical trials of mutant 471 can be initiated, since the correlation between activity in bioassays selected in this study and the results of clinical studies of wild-type TNF has not been established. In clinical trials of wild-type recombinant TNF, hypotension was the dose-limiting factor (Creaven *et al.*, 1987; Kimura *et al.*, 1987). We are currently investigating the hypotensive effect of mutant 471 in rats and dogs.

ACKNOWLEDGEMENTS

We thank Dr. M. Kawakami for his kind introduction to the cachectin assay and for the gift of 3T3-L1 cell, Dr. M. Saito and Ms. N. Matsui for their comments on the *in vivo* anti-tumor assay, and Ms. Y. Nakazato and Ms. K. Wakabayashi for their secretarial assistance.

REFERENCES

BEUTLER, B. and CERAMI, A., Cachectin and tumor necrosis factor as two sides of the same biological coin. *Nature (Lond.)*, **320**, 584–588 (1986).

BEUTLER, B., GREENWALD, D., HULMES, J.D., CHANG, M., PAN, Y.-C.E., MATHISON, J., ULEVITCH, R. and CERAMI, A., Identity of tumor necrosis factor and the macrophage-secreted factor cachectin. *Nature (Lond.)*, **316**, 552–554 (1985).

[illegible], M., SHERWIN, S.A., ROSENBLUM, M. and GUTTERMAN, J., Phase [illegible] of recombinant tumor necrosis factor in cancer patients. *Cancer Res.*, **47**, 2986–2989 (1987).

CARSWELL, E.A., OLD, L.J., KASSEL, R.L., GREEN, S., FIORE, N. and WILLIAMSON, B., An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. nat. Acad. Sci. (Wash.)*, **72**, 3666–3670 (1975).

CREASEY, A.A., DOYLE, L.V., REYNOLDS, M.T., JUNG, T., LIN, L.S. and VITT, C.R., Biological effects of recombinant human tumor necrosis factor and its novel muteins on tumor and normal cell lines. *Cancer Res.*, **47**, 145–149 (1987).

CREAVEN, P.J., PLAGER, J.E., DUPERE, S., HUBEN, R.P., TAKITA, H., MITTELMAN, A. and PROEFROCK, A., Phase I clinical trial of recombinant [illegible]n tumor necrosis factor. *Cancer Chemother. Pharmacol.*, **20**, 137–[illegible]1987).

FRAKER, P.J. and SPECK, J.C., Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. biophys. Res. Comm.*, **80**, 849–857 (1978).

GATANAGA, T., NOGUCHI, K., TANABE, Y., INAGAWA, H., SOMA, G. and MIZUNO, D., Antitumor effect of systemic administration of novel recombinant tumor necrosis factor (rTNF-S) with less toxicity than conventional rTNF-α *in vivo*. *J. biol. Resp. Modif.*, **8**, 278–286 (1989).

HAVELL, E.A., FIERS, W. and NORTH, R.J., The antitumor function of tumor necrosis factor (TNF). I. Therapeutic action of TNF against an established murine sarcoma is indirect, immunologically dependent, and limited by severe toxicity. *J. exp. Med.*, **167**, 1067–1985 (1988).

[illegible]O, R., TAKEDA, K., NAGUMO, M., KONNO, K., HASEGAWA, A., [illegible]A, K. and IKEHARA, M., Induction of differentiation of human monoblastic and myeloblastic leukemia cell lines by TNF muteins. *Biochem. biophys. Res. Comm.*, **160**, 820–827 (1989).

KAWAKAMI, M., MURASE, T., OGAWA, H., ISHIBASHI, S., MORI, N., TAKAKU, F. and SHIBATA, S., Human recombinant TNF suppresses lipoprotein lipase activity and stimulates lipolysis in 3T3-L1 cells. *J. Biochem. (Tokyo)*, **101**, 331–338 (1987).

KAWAKAMI, M., PEKARA, P.H., LANE, M.D. and CERAMI, A., Lipoprotein lipase suppression in 3T3-L1 cells by an endotoxin-induced mediator from exudate cells. *Proc. nat. Acad. Sci. (Wash.)*, **79**, 912–916 (1982).

KIMURA, K., TAGUCHI, T., URUSHIZAKI, I., OHNO, R., ABE, O., FURUE, [illegible], HATTORI, T., ICHIHASHI, H., INOGUCHI, K., MAJIMA, H., NIITANI, [illegible]TA, K., SAITO, T., SUGA, S., SUZUOKI, Y., WAKUI, A., YAMADA, [illegible] and the A-TNF COOPERATIVE STUDY GROUP, Phase-I study of recombinant human tumor necrosis factor. *Cancer Chemother. Pharmacol.*, **20**, 223–229 (1987).

LAEMMLI, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*, **227**, 680–685 (1970).

NAKAMURA, S., MASEGI, T., FUKUOKA, M., KITAI, K., KATO, A., ICHIKAWA, Y., WATANABE, N. and NIITSU, Y., Interactions between novel tumor necrosis factor-α mutants and receptors on tumor and normal cells. *Agric. biol. Chem.*, **55**, 53–57 (1991).

NAKAMURA, S., MASEGI, T., KITAI, K., ICHIKAWA, Y., KUDO, T., AONO, R. and HORIKOSHI, K., Extracellular production of human tumor necrosis factor-α by *Escherichia coli* using a chemically-synthesized gene. *Agric. biol. Chem.*, **54**, 3241–3250 (1990).

NAKAMURA, S., MASEGI, T., KITAI, K., KUDO, A., WATANABE, T. and ICHIKAWA, Y., Production of the human immunoglobulin γ_1 chain constant region polypeptides in *Escherichia coli*. *J. Biotechnol.*, **8**, 141–148 (1988).

NIITSU, Y., WATANABE, N., UMENO, H., SONE, H., NEDA, H., YAMAUCHI, N., MAEDA, M. and URUSHIZAKI, I., Synergistic effects of recombinant tumor necrosis factor and hyperthermia on *in vitro* cytotoxicity and artificial metastasis. *Cancer Res.*, **48**, 654–657 (1988*a*).

NIITSU, Y., WATANABE, N., YAMAUCHI, N., MAEDA, M., KOGAWA, K., KURIYAMA, H., NEDA, H. and SONE, H., The anti-metastatic effect of tumor necrosis factor on the artificial metastasis of highly and low-metastatic clones derived from methylcholanthrene induced-A fibrosarcoma cells. *Cancer J.*, **2**, 91–94 (1988*b*).

NORTH, R.J. and HAVELL, E.A., The antitumor function of tumor necrosis factor (TNF). II. Analysis of the role of endogenous TNF in endotoxin-induced hemorrhagic necrosis and regression of an established sarcoma. *J. exp. Med.*, **167**, 1086–1099 (1988).

OLD, L.J., Tumor necrosis factor (TNF). *Science*, **230**, 630–632 (1985).

PENNICA, D., NEDWIN, G.E., HAYFLICK, J.S., SEBURG, P.H., DERYNCK, R., PALLADINO, M.A., KOHR, W.J., AGGARWAL, B.B. and GOEDDEL, D.V., Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature (Lond.)*, **312**, 724–729 (1984).

REGENASS, U., MULLER, M., CURSCHELLAS, E. and MATTER, A., Anti-tumor effects of tumor necrosis factor in combination with chemotherapeutic agents. *Int. J. Cancer*, **39**, 266–273 (1987).

SHIRAI, T., YAMAGUCHI, H., ITO, H., TODD, C.W. and WALLACE, R.B., Cloning and expression in *Escherichia coli* of the gene for human tumor necrosis factor. *Nature (Lond.)*, **313**, 803–806 (1985).

SOMA, G., KITAHARA, N., TSUJI, Y., KATO, M., OSHIMA, H., GATANAGA, T., INAGAWA, H., NOGUCHI, K., TANABE, Y. and MIZUNO, D., Improvement of cytotoxicity of tumor necrosis factor (TNF) by increase in basicity of its N-terminal region. *Biochem. biophys. Res. Comm.*, **148**, 629–635 (1987).

TALMADGE, J.E., TRIBBLE, H.R., PENNINGTON, R.W., PHILLIP, H. and WILTROUT, R.H., Immunomodulatory and immunotherapeutic properties of recombinant γ-interferon and recombinant tumor necrosis factor in mice. *Cancer Res.*, **47**, 2563–2570 (1987).

WATANABE, N., NIITSU, Y., UMENO, H., KURIYAMA, H., NEDA, H.,

YAMAUCHI, N., MAEDA, M. and URUSHIZAKI, I., Toxic effect of tumor necrosis factor on tumor vasculature in mice. *Cancer Res.*, **48**, 2179–2183 (1988*a*).

WATANABE, N., NIITSU, Y., UMENO, H., SONE, H., NEDA, H., YAMAUCHI, N., MAEDA, M. and URUSHIZAKI, I., Synergistic cytotoxic and antitumor effects of recombinant human tumor necrosis factor and hyperthermia. *Cancer Res.*, **48**, 650–653 (1988*b*).

WATANABE, N., NIITSU, Y., YAMAUCHI, N., UMENO, H., SONE, H., NEDA, H. and URUSHIZAKI, I., Antitumor synergism between recombinant human tumor necrosis factor and recombinant human interferon-γ. *J. biol. Resp. Modif.*, **7**, 24–31 (1988*c*).

YAMAUCHI, N., KURIYAMA, H., WATANABE, N., NEDA, H., MAEDA, M. and NIITSU, Y., Intracellular hydroxyl radical production induced by recombinant tumor necrosis factor and its implication in the killing of tumor cells *in vitro*. *Cancer Res.*, **49**, 1671–1675 (1989).

YAMAUCHI, N., WATANABE, N., KURIYAMA, H., NEDA, H., MAEDA, M., HIMENO, T., TSUJI, Y. and NIITSU, Y., Suppressive effects of intracellular glutathione on hydroxyl radical production induced by tumor necrosis factor. *Int. J. Cancer*, **46**, 884–888 (1990).

YONE, K, SUZUKI, J., TSUNEKAWA, N., ICHIKAWA, Y. and FURUKAWA, S., Detection of TNF in blood from patients with Kawasaki syndrome. *Immunobiology*, **175**, 106 (1987).

Eur. J. Biochem. 220, 771–779 (1994)

Human tumor necrosis factor mutants with preferential binding to and activity on either the R55 or R75 receptor

Xaveer VAN OSTADE[1], Peter VANDENABEELE[1], Jan TAVERNIER[2] and Walter FIERS[1]

[1] Laboratory of Molecular Biology, Gent University, Belgium
[2] Roche Research Gent, Belgium

(Received October 13/December 20, 1993) – EJB 93 1549/3

Previously, we reported that the cytotoxic activity of human (h) tumor necrosis factor (TNF) on murine (m) L929 cells requires the integrity of three loops (positions 30–36, 84–88 and 138–150) which cluster around the interface between each two subunits of the trimeric hTNF structure. The collection of hTNF mutants was further characterized by their activity on various human cell systems as well as by their binding to the two types of hTNF receptor (R), R55 and R75. It turned out that two amino acids (Leu29 and Arg32) were specifically involved in hR75 binding, as Leu29→Ser (L29S) and Arg32→Trp (R32W) mutant molecules had largely lost binding to hR75, but not to hR55. In order to screen for more highly R55-specific mutants, nine other amino acids were inserted at these two positions; only the substitutions L29G and L29Y showed an increased differential binding as compared to L29S, while no further improvement was found with mutations at position 32 compared to R32W. Biological assays mediated either by hR55 or hR75 confirmed the results obtained by physical binding to purified receptors. A similar substitution in mTNF, Arg32→Tyr, also resulted in a preferential loss of binding to hR75 and a large decrease in mR75-mediated bioactivity. Except for the double mutant L29S-R32W, all other tested amino acid substitutions in the loops at positions 30–36 or 84–88 of hTNF led to a substantial loss of affinity for both receptors and a concomitant reduction of biological activity. In the loop at positions 138–150, the non-conservative replacement of Glu by Lys at position 146 (E146K) resulted in an even lower binding to R75 as compared to R32W, while binding on and bioactivity through R55 was only slightly reduced. Remarkably, a reversed differential binding was observed after substitution at position 143 in hTNF; replacing Asp by non-conservative residues such as Tyr, Phe or Asn resulted in a much larger decrease in binding to R55 than to R75. In conclusion, receptor-specific mutants such as R32W, E146K and D143N can be used to study the function either of R55 or R75 on different human cell types. *In vivo*, we presume that the R55-specific mutants will retain antitumor activity in the absence of R75-dependent, severe side effects.

Tumor Necrosis Factor (TNF) is a cytokine primarily produced by activated macrophages and was first discovered by its ability to cause hemorrhagic necrosis of tumors *in vivo* as well as its cytotoxic activity on malignant cells *in vitro* (reviewed in Fiers, 1993). However, administration of murine (m) TNF as an antitumor agent revealed a high *in vivo* toxicity in mice (Brouckaert et al., 1986; Cerami and Beutler, 1988; Brouckaert et al., 1992) and injection of high human (h) TNF doses in humans, necessary for tumor regression, caused serious side effects, such as hypotension and hepatotoxicity (Spriggs and Yates, 1992). This toxicity most probably reflects the pleiotropic character of TNF and its important role in inflammatory and immune responses (Fiers, 1993).

In man and mouse, two different types of TNF receptor (R), R55 (55 kDa) and R75 (75 kDa), were cloned and have been shown to be present on a wide variety of cell types (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990). Specific clustering of R55 by agonistic anti-TNF-R allowed several TNF activities to be mimicked, such as cytotoxicity, fibroblast proliferation, interleukin-6 induction and prostaglandin E_2 synthesis (Espevik et al., 1990; Engelmann et al., 1990; Tartaglia et al., 1991). Evidence for a signal-transducing role of R75 was demonstrated in T-cell systems, such as thymocyte proliferation (Tartaglia et al., 1991) and induction of granulocyte-macrophage-colony-stimulating factor (GM-CSF; Vandenabeele et al., 1992), again by means of specific, agonistic antibodies. However, the use of neutralizing monoclonal antibodies against either type of receptor revealed the existence of a certain degree of redundancy for R55 and R75, showing a partial involvement of R75 in presumed R55-mediated activities (Shalaby et al., 1990; Hohmann et al., 1990). To reconcile the separate and redundant features of both receptors, Tartaglia and colleagues (1992 and 1993) proposed a model in which R75 plays an indirect role in R55 responses by binding TNF and delivering it to the lower affinity R55.

Correspondence to W. Fiers, Laboratory of Molecular Biology, Gent University, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium
Fax: +32 9 2645348.

Abbreviations. GM-CSF, granulocyte macrophage colony-stimulating factor; h, human; m, murine; R, receptor; TNF, tumor necrosis factor; wt, wild-type.

On the basis of mutation studies, we presented previously an outline of the TNF-receptor-binding site (Van Ostade et al., 1991). Combination of random and site-specific mutagenesis with a screening procedure, based on the activity on murine L929 cells, revealed seven relevant mutations, located in or nearby three loops (positions 30–36, 84–88 and 138–150) which surround the cleft between two subunits, at the broader half of the pyramidally shaped, trimeric molecule (Sprang and Eck, 1992). Similar results were reported by other groups (Yamagishi et al., 1990; Zhang et al., 1992). Hence, a TNF molecule has three receptor-binding sites, in agreement with its mechanism of action which involves receptor clustering (Loetscher et al., 1991; Pennica et al., 1992).

Recently, we reported on two hTNF mutants, Leu29→Ser and Arg32→Trp (L29S and R32W, respectively), which maintained their binding to R55, but showed a considerable reduction in affinity for R75 (Van Ostade et al., 1993). In addition, this difference in binding was reflected in the biological activities, mediated by R55 or R75, such as cytotoxicity to HEp-2 cells and lack of GM-CSF induction in hTNF-R75-transfected PC60 cells, respectively. Moreover, the *in-vivo* antitumoral activity of R32W in xenografted nude mice was similar to that of wild-type (wt) hTNF. Since, in mice, the toxicity of mTNF, which binds to both mR55 and mR75, is considerably higher than hTNF, which binds only to mR55 (Lewis et al., 1991; Tartaglia et al., 1991), it may be concluded that R75 plays a special role in the severe systemic toxicity of TNF (Brouckaert et al., 1992). Therefore, the hR55-specific mutants, which are expected to behave in the human system as the equivalent of hTNF in mice, might be more specifically antitumor-directed agents without R75-dependent side effects. In the present study we have characterized a series of TNF mutants which differentially bind to and act on the two hTNF receptor types.

EXPERIMENTAL PROCEDURES

Cell cultures

Human HEp-2 and KYM cells as well as murine CT6, WEHI and PC60-hTNF-R75$^+$ cells were grown in RPMI 1640 medium, supplemented with 10% (by vol.) inactivated fetal calf serum, L-glutamine (2 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (50 μM), penicillin G (50 U/ml) and streptomycin sulfate (50 μg/ml). L929 cells were grown in Dulbecco's medium, supplemented with 10% inactivated newborn calf serum. GM-CSF-dependent FDCp1 cells were grown in the same medium, supplemented with 10% WEHI-3 supernatant as a source of murine interleukin-3.

Assays for cytotoxic activity

Evaluation of the cytotoxic activity of hTNF and its muteins on murine cells was determined in a 20-h assay on actinomycin-D-treated (1 μg/ml) WEHI 164 clone 13 cells (Espevik and Nissen-Meyer, 1986) and L929 cells (Ruff and Gifford, 1981), both murine fibrosarcoma cell lines. Cytotoxic activity on human cells was determined on CHX (50 μg/ml)-treated HEp-2 cells, a human larynx carcinoma, and actinomycin-D-treated KYM-39A6 cells, a human rhabdomyosarcoma cell line (kindly provided by Dr A. Meager; Meager, 1991). In case of WEHI 164 clone 13, L929 and KYM-39A6 cells, the number of surviving cells was quantified by the colorimetric MTT assay (Mosmann, 1983) and, in the case of HEp-2 cells, by crystal violet staining, as described previously (Fransen et al., 1986).

Assay for GM-CSF induction

Previously, we described a biological TNF assay, which is mainly hR75 mediated, using PC60 cells (a rat/mouse T-cell hybridoma), transfected with hR75 cDNA (Vandenabeele et al., 1992; Van Ostade et al., 1993). In contrast to parental PC60 cells, these hR75$^+$ PC60 cells respond to hTNF by induction of GM-CSF production, which is synergized by the addition of interleukin-1. Briefly, 3×10^4 hR75$^+$ cells/microtiter well were incubated for 36 h in the presence of a constant concentration of recombinant human interleukin-1β (1 ng/ml) and serial dilutions of hTNF or its muteins. The amount of secreted GM-CSF was quantified by the proliferation of FDCp1 cells (Vandenabeele et al., 1990). Neither TNF nor its mutants interfered in the GM-CSF assay.

T-cell proliferation assay

Growth induction on CT6 cells (kindly provided by Dr M. Palladino and Dr. M. Feldmann) was performed as described (Ranges et al., 1989). Briefly, 5×10^3 cells/well were incubated with wt or Δ7-R32Y mTNF. After incubation for 18–24 h, the assay plates were pulsed with 1 μCi/well [^{3}H]thymidine deoxyribose, and incubated further for 4 h. Then the cells were harvested and incorporated [^{3}H]-thymidine deoxyribose was measured in a scintillation counter.

Binding of TNF to recombinant hR55 and hR75

Binding assays were carried out by Dr. H. Loetscher (Hoffmann-La Roche) as previously described (Loetscher et al., 1991; Van Ostade et al., 1993). Briefly, hR55-IgGγ3 and hR75-IgGγ3 fusion proteins (0.1 μg/ml and 0.3 μg/ml, respectively; Lesslauer et al., 1991), were added to microtiter plates and left overnight at 4°C. Blocking was with 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.02% NaN_3 and 1% defatted milk powder. The microtiter plates were then washed with NaCl/P_i (0.15 M NaCl/0.01 M P_i, pH 7.5) and incubated with 10 ng/ml ^{125}I-labeled wtTNF in the presence of increasing concentrations of wt TNF or mutant TNF, ranging over 10^{-2}–10^{-5} μg/ml. The total volume was 100 μl/well and each concentration was assayed in triplicate. After 3 h at room temperature, the wells were thoroughly washed with NaCl/P_i, and bound radioactivity was measured in a γ-counter. The concentration at which the competitor (e.g. mutant TNF) reduced the specific binding by 50% was determined, and used for comparison with wt TNF as competitor.

Mutagenesis of human and murine DNA

The hTNF expression cassette was subcloned into pMa and pMc phasmids as described (Van Ostade et al., 1991), resulting in a system suitable for mutagenesis and expression in bacteria. mTNF, preceded by the Trp promoter, was subcloned from the bacterial expression plasmid pAT153mTNF into pMa and pMc; after mutagenesis, mTNF mutants were cloned back into the pAT153 vector in order to obtain a high expression level of mutant mTNF. Site-specific mutagenesis was carried out following the procedure described by Stanssens et al. (1989). In order to rapidly check for the presence

of a mutation, oligonucleotides were synthesized, in which codon mutations were accompanied by destruction or formation of a restriction site. The following oligonucleotides were used (underlined bases represent mutations):

L29S-R32W	5′GGGCATTGGCCCAGCGGTTGGACCACTG-GAGC3′ (+Ava2)
A33P	5′GGGCATTGGGCCGGCGG3′ (+Nae1)
Δ31-35+GSG	5′CGCCATTGGCCAGGAGGCCTGAACCGTT-CAGCCACTGGAGC3′ (+Stu1)
L29T	5′CCGGCGGTTGGTCCACTGGAGC3′ (+Ava2)
L29E	5′CGCCATTGGCCCGACGGTTTTCCCACTG-GAGC3′ (−Nci1)
R32A	5′GGGCATTGGCCGCGCGGTTCAGCC3′ (−Hpa2)
D143Y	5′CGGCAAAGTACAGATAGTCGG3′ (+Rsa1)
D143F	5′CTCGGCAAAGAAGAGATAGTCG3′ (−Taq1)
D143E	5′CTCGGCAAACTCGAGATAGTCG3′ (+Xho1)
D143N	5′CTCGGCAAAATTGAGATAGTCG3′ (−Taq1)
E146Q	5′GACCTGCCCACTGTCGGCAAAGTCGAGA-TAG3′ (−Hinf1)
E146H	5′CCTGCCCAGAGTGGGCAAAGTCG3′ (−Hinf1)

The double mutants R32W-E146Q, R32W-E146H and R32W-E146K were constructed by performing site-specific mutagenesis as described above, using the E146 oligonucleotides and the R32W mutant TNF gene as a template.

In order to obtain random substitutions of amino acids at positions 29 and 32, we synthesized two oligonucleotides, 5′CCACGCCATTCGCGAGGAGGGCATTGGCCC GGCGGTTXXXCCCTGGAGC3′ and 5′CCACGCCAT TCGCGAGGAGGGCATTGGCXXXGCGGTTCAGCC3′, which contained a degenerated codon at either of these two positions, together with the introduction of a unique *Nru*1 site. This allowed us to select for mutation-bearing phasmids by digesting the DNA pool (resulting after transformation of the hybridization mixture to the WK6 mutS strain) with *Nru*1. The linear fragment was isolated, ligated and transformed to the *Escherichia coli* SURE strain (Stratagene Cloning Systems).

The muteins L29S, R32W, L36F, A84V, S86F, V91A, V91D and E146K were constructed by random or site-directed mutagenesis, as described previously (Van Ostade et al., 1991).

Screening for inactive mutants

The transformants that were obtained after site-directed mutagenesis were screened by colony hybridization; positive colonies were checked for the presence or absence of the restriction site which accompanied the desired mutation, and were then used for biological-activity tests.

Colonies, resulting from the random mutagenesis procedure, were inoculated into the wells of a microtiter plate and grown overnight at 37°C. Cells were disrupted by the addition of lysozyme and five freeze/thaw cycles. After centrifugation and polyethylene-imine (0.4%) precipitation, the supernatant was diluted several times and tested for L929 and HEp-2 cytotoxic activity as described previously (Van Ostade et al., 1991). On the basis of their different behavior in these assays, several classes of mutants could be distinguished. As a result, 16 (position 29) and 20 (position 32) clones were retained for sequence analysis. We finally characterized eight different replacements (including the wt amino acid) at position 29 and ten at position 32, together with the R32V-A33P mutant which was generated as an artefact of the procedure. In addition, the wt amino acids were defined by another codon at each position (TTG instead of CTG for Leu29 and CGT instead of CGG for Arg32) and thus were clearly generated by the random mutagenesis. As a result, they could serve as an intrinsic control of the mutagenesis procedure and indeed showed a wt behavior, as tested by L929 and HEp-2 cytotoxicity (data not shown). All mutants described here showed a similar expression level and solubility in *E. coli*, as compared to wt TNF.

Purification of the muteins

Since all mutants were highly soluble in the bacterial supernatants, polyethylene-imine precipitation and ammonium-sulfate fractionation of the bacterial lysates was sufficient to produce 30–70% purity, as determined by SDS/PAGE. Hence, the mutants Δ31–35+GSG, A33P, L29T, L29E, R32A, D143Y, D143F, D143E, D143N, E146Q, E146H, R32W-E146Q, R32W-E146H and R32W-E146K were analyzed in the binding and biological assays. All other muteins were further purified using a Mono-Q chromatography column (Pharmacia P-L Biochemicals) to a purity of >90%. Only the wt was further purified by means of an additional Mono-S (Pharmacia P-L Biochemicals) chromatography step. Conditions for chromatography are described in Tavernier et al. (1990).

RESULTS AND DISCUSSION

Analysis of mutations in the loop at positions 30–36

By random mutagenesis and subsequent biological screening on L929 cells, we found seven amino acids to be directly or indirectly involved in the active center of hTNF (Van Ostade et al., 1991). Among these, Leu29 and Arg32 were shown to specifically interact with hR75, as the L29S and R32W muteins had 100–500-times reduced affinity for hR75, but not for hR55 (Van Ostade et al., 1993). Next, we tested whether other amino acid substitutions at these positions would result in a more pronounced differential binding. Therefore, we used oligonucleotides degenerated either at the codon for amino acid Leu29 or Arg32 and obtained seven and nine amino acid replacements at positions 29 and 32, respectively. Other substitutions were generated by site-specific mutagenesis including: L29E, L29T, R32A and the double-mutation L29S-R32W. All mutants were tested for competition binding to the two recombinant human receptors and were assayed for cytotoxic activity on two human (HEp-2 and KYM) and two murine (L929 and WEHI) cell lines. Cytotoxicity is an R55-mediated process, both in human and in murine systems. Indeed, selected monoclonal antibodies directed against hR55, or polyclonal antibodies directed against mR55 show cytotoxic activity on human HEp-2 and KYM cells (P. Vandenabeele, unpublished results) and on murine fibrosarcoma cells (Tartaglia et al., 1991), respectively, while agonistic anti-R75 are not. However, these anti-hR75 monoclonal or anti-mR75 polyclonal anti-

Table 1. Relative binding constants and specific biological activities of selected TNF mutants. Binding constants to the two types of human receptors were determined by competition assay (see Experimental Procedures). The results are presented taking the wt value as 100%. Specific biological activities as assayed by cytotoxicity are mediated by R55; cytotoxicity was determined on human HEp-2 or KYM cells, as well as on murine L929 and WEHI cells. The wtTNF reference had a specific activity of 1×10^8 U/mg. The values for the cytotoxic activity, relative to wt TNF, are based on the average result on HEp-2 and KYM cells for the hR55-mediated effects, and on L929 and WEHI cells for the mR55-mediated effects, respectively. Biological activity mediated by hR75 was assayed on the basis of induction of GM-CSF (see Experimental Procedures); the values are given both as the concentration of TNF (mutant) required to induce 25% of the maximal secretion of GM-CSF obtained with wt hTNF, as well as the amount of wt hTNF activity. Biological activity mediated by mR75 was assayed on the basis of proliferation of CT6 cells. n.d. = not detectable. The variation between independent cytotoxicity assays was < 30%, in the binding assays < 50% and in the GM-CSF induction assays < 50%. In all those tests, it was observed that the higher the specific activity of TNF mutants, the lower was the variation between the results from independent experiments.

TNF mutant	Relative binding constant		Biological activity for								
	human		human					murine			mR75-mediated
	R55	R75	hR55-mediated			hR75-mediated		mR55-mediated			
			HEp-2	KYM		PC60 (hR75$^+$)		L929	WEHI		CT6
Human	%		U/mg		%	ng/ml	%	U/mg		%	U/mg
Wt	100	100	1×10^8	1×10^8	100	2	100	1×10^8	1×10^8	100	
Loop 30–36											
L29S	12	0.9	2.7×10^7	4.9×10^6	16	200[a]	1	2.2×10^5	1.4×10^5	0.18	
L29G	27	0.2	2.4×10^7	1.6×10^7	20	10[a]	20.5	7.2×10^5	1.8×10^6	1.26	
L29Y	33	0.1	2.2×10^7	1.6×10^7	19	16[a]	12.5	1×10^6	1.8×10^6	1.4	
R32W	69	0.2	1.2×10^8	3.8×10^8	250	400[a]	0.5	2.1×10^5	4.1×10^5	0.31	
R32V	20	7.5	4.6×10^7	3.9×10^7	42	4	50	5.5×10^7	8×10^7	63	
R32Y	40	0.4	3.7×10^7	1×10^8	68	10	20	1.8×10^7	4.1×10^7	29	
L29S-R32W	40	0.04	4.7×10^7	2.9×10^7	38	10 000[a]	0.02	3.3×10^4	1×10^6	0.07	
Loop 84–88											
A84V	0.07	n.d.	1.5×10^4	9×10^3	0.01	n.d.	0	$<1\times10^5$	$<1\times10^5$	<0.01	
Loop 138–150											
D143Y	<0.02	6	$<10^3$	2×10^6	<0.01	20[a]	10	$<10^5$	$<10^6$	<0.01	
D143F	<0.02	3	$<10^3$	6.5×10^6	<0.01	8[a]	25	$<10^5$	$<10^6$	<0.01	
D143E	0.3	15	2×10^5	3×10^5	0.25	4[a]	50	$<10^5$	$<10^6$	<0.01	
D143N	<0.04	15	$<10^3$	2×10^4	<0.01	4[a]	50	$<10^5$	$<10^6$	<0.01	
E146Q	200	214	1.8×10^8	3×10^8	240	1	200	7×10^7	2×10^8	135	
E146H	87	5	8×10^7	4.8×10^7	64	20[a]	10	6.4×10^3	2.5×10^4	<0.01	
E146K	50	0.03	4×10^7	3.5×10^7	37	n.d.	0	$<1\times10^5$	$<1\times10^6$	<0.01	
Combinations											
R32W-E146Q	74	0.3	2.1×10^8	1.8×10^8	195	24[a]	8.3	5×10^5	7×10^5	0.6	
R32W-E146H	6	<0.03	1.6×10^7	1.2×10^7	14	n.d.	0	1.3×10^3	2.5×10^4	<0.01	
R32W-E146K	6	n.d.	1.1×10^7	3.8×10^6	7.4	n.d.	0	$<1\times10^5$	$<1\times10^6$	<0.01	
Murine											
wt	118	56							1×10^8		1×10^8
Δ7-R32Y	15	<0.03							3.3×10^7		1.4×10^6

[a] In the dose/response curve, the maximum plateau level of GM-CSF induction was substantially lower (<50%) than the level obtained with wt hTNF.

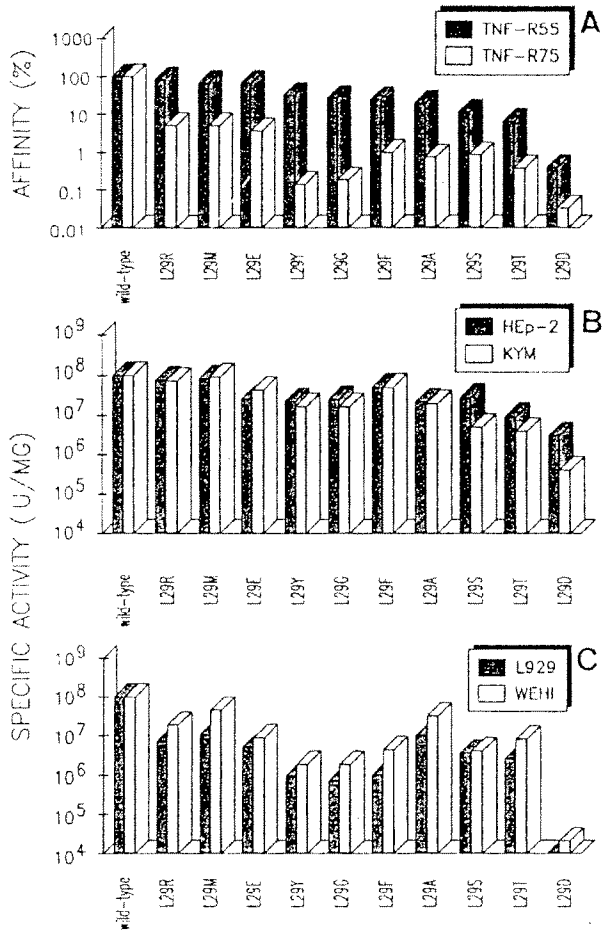


Fig. 1. Relative physical binding mediated by the two types of hTNF-R and biological activities for TNF mutants at position 29. Leu in wt hTNF has been replaced by different amino acids as indicated below the abscissa. (A) Affinities (taking the wt value as 100%) for hR55 and hR75 as determined by competition binding. Mutants are shown in order of decreasing R55 affinity. (B) Specific activities (wt TNF = 1×10^8 U/mg) on the human cell lines HEp2 and KYM (mediated totally or largely by hR55). (C) Specific activities (wt TNF = 1×10^8 U/mg) on the murine cell lines L929 and WEHI (mediated totally or largely by mR55).

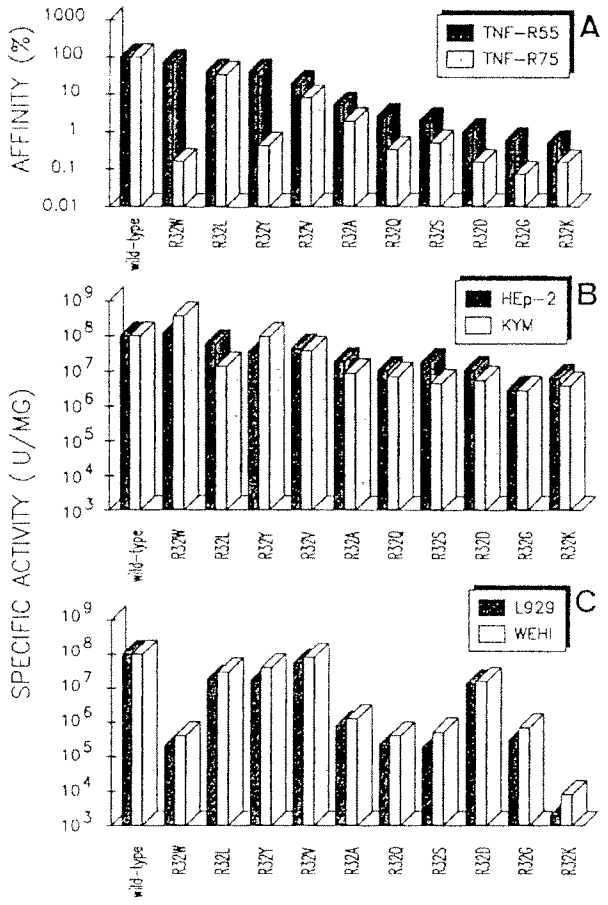


Fig. 2. Relative physical binding mediated by the two types of hTNF-R and biological activities for TNF mutants at position 32. Arg in wt hTNF has been replaced by different amino acids as indicated below the abscissa. Further details are as indicated in the legend to Fig. 1.

bodies are active in the induction of GM-CSF in hR75-transfected PC60 cells, a rat/mouse T-cell hybridoma (Vandenabeele et al., 1992), and in a proliferation assay of CT6 cells, a murine T-cell line (Tartaglia et al., 1991), respectively. Hence, the latter assay systems allow one to test for R75-mediated effects. Exact quantification of GM-CSF induction in the aforementioned hR75-transfected PC60 cells, however, was often difficult, as several muteins resulted in different shapes of dose/response curves with various levels of maximal GM-CSF induction. As the maximal level induced by most muteins was substantially lower than those by wt hTNF, a comparison on the basis of 50% of the maximal wt hTNF activity was not feasible. Therefore, we present the R75-mediated bioactivity data as the amount of mutant TNF required to induce 25% of the maximal level of GM-CSF obtained with wt hTNF. From these data, normalized values were calculated (Table 1).

For hR55, there is a close correlation between the relative values obtained from the solid-phase competition-binding tests and the hR55-specific bioassays (cytotoxicity for KYM or HEp-2 cells).

The data (Figs 1 and 2; Table 1) indicate that, compared to R32W, no amino acid substitution could be found which resulted in a more drastic loss of binding to R75, while maintaining binding to and bioactivity on R55. However, all replacements at position 29 showed similar or even better (L29G, L29Y) differential binding characteristics, relative to L29S. The double-mutation L29S-R32W (Fig. 3) showed no further marked affinity reduction for hR75 and binding on hR55 was slightly decreased.

Remarkably, the cytotoxicity of these mutants for the murine cell lines was, in the majority of cases, considerably more affected compared to the human cell lines, indicating a more stringent conformational restriction for h/mR55 interaction than for the homologous, intraspecies interaction. In contrast, it is noteworthy that in those cases where hTNF muteins (L29G, L29Y, R32V, R32Y) retained above 1% of the cytotoxic activity on L929 and WEHI cells and showed a reduced hR75 binding (7.5% or less), the biological activity on the hR75-transfected PC60 cells still reached fairly high levels (Table 1). This suggests a cooperation between the transfected hR75 and the endogenous mR55 in GM-CSF induction, as also observed in PC60 cells transfected with both hTNF receptors (P. Vandenabeele et al., unpublished results).

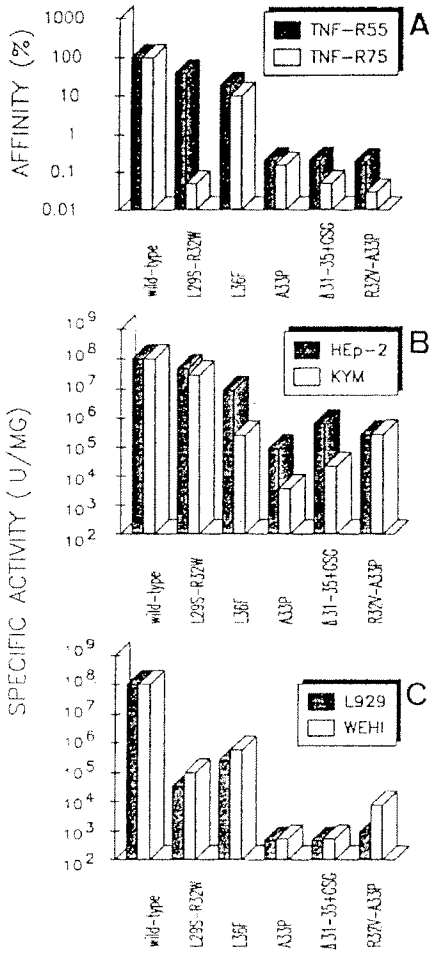


Fig. 3. Relative physical binding mediated by the two types of hTNF-R and biological activities for TNF mutants in the loop at positions 30–36 in hTNF. Further details are as indicated in the legend to Fig. 1.

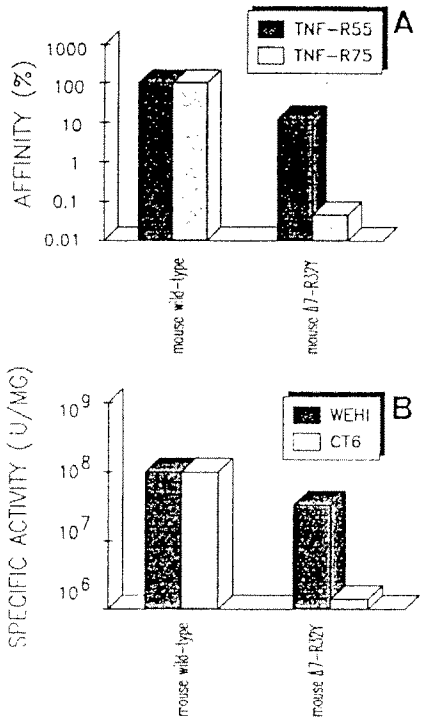


Fig. 4. Relative physical binding mediated by the two types of hTNF-R and biological activities for the Δ7-R32Y mTNF mutant. (A) Relative affinities (wt mTNF = 100%) for hR55 and hR75. (B) Specific activities (wt mTNF = 1×10^7 U/mg) on WEHI and CT6 cells (the latter response is mR75 mediated).

We can conclude that the induction of GM-CSF in PC60 cells is exclusively mediated by the transfected hR75 only when the hTNF mutants show a considerably reduced affinity for mR55 (less than 1% activity remaining), as is the case for most of the other muteins.

In the next series of experiments, we introduced more drastic mutations that could disturb the conformation of the loop at positions 29–36 and hence influence R75 binding (Fig. 3). Replacement of amino acids Arg31–Ala35 by a Gly-Ser-Gly stretch resulted in a marked reduction of R55 binding. Also, the substitution of Ala33 by the rotation constrained Pro residue (alone or in combination with R32V), induced a local conformational rearrangement such that sites involved in R55 interaction were likewise affected. The substitution of Leu by the bulky side chain of Phe on the more distant position 36, reduced R55 and R75 affinity only moderately (by a factor of 5 and 10, respectively).

Among the two mutants which maintained a relatively high activity on the murine L929 and WEHI cells, i.e. mR55-mediated bioactivity, R32Y showed the most profound reduction in hR75 affinity. Therefore, in order to develop an mR55-specific mutant, a similar mutation was introduced in mTNF. This mutation was combined with a deletion of the seven N-terminal amino acids of mTNF. In the case of hTNF, the latter change resulted in a threefold higher cytotoxic activity on L929 cells (Creasey et al., 1987; Nakamura et al., 1991a, 1991b; Sidhu and Bollon, 1989; our own unpublished results). It was hoped that the N-terminal Δ7 deletion would compensate for a possible decrease in mR55 affinity. However, no difference in cytotoxic activity on WEHI, L929 or KYM cells was found between Δ7-R32Y-mTNF and R32Y-mTNF (data not shown), indicating that this N-terminal deletion in the case of mTNF does not result in enhanced cytotoxicity. The Δ7-R32Y mutant showed about a 10-fold reduction in binding to hR55, although activity on WEHI cells was only threefold decreased. Affinity for hR75, however, was drastically affected, but growth-stimulating activity on the murine CT6 T-cell line (which is an mR75-mediated event; Tartaglia et al., 1991) was only reduced by a factor of approximately 25 (Fig. 4; Table 1). Once again, these results illustrate the more stringent restriction on heterologous ligand-receptor binding as compared to homologous intra-species interaction.

Analysis of mutations in the loop at positions 84–88

From cytotoxicity studies, it was previously concluded that mutations in the loop at positions 84–88 induced a marked decrease in activity on murine L929 cells (Van Ostade et al., 1991). As expected, alterations in this region (A84V, S86F, V91A and V91D) strongly decreased interaction with R55. Furthermore, in the case of A84V, hR75 binding and bioactivity were even undetectable (Fig. 5; Table 1).

Analysis of mutations in the loop at positions 138–150

The non-conservative E146H and E146K replacements induced a very pronounced differential binding (reduced R75

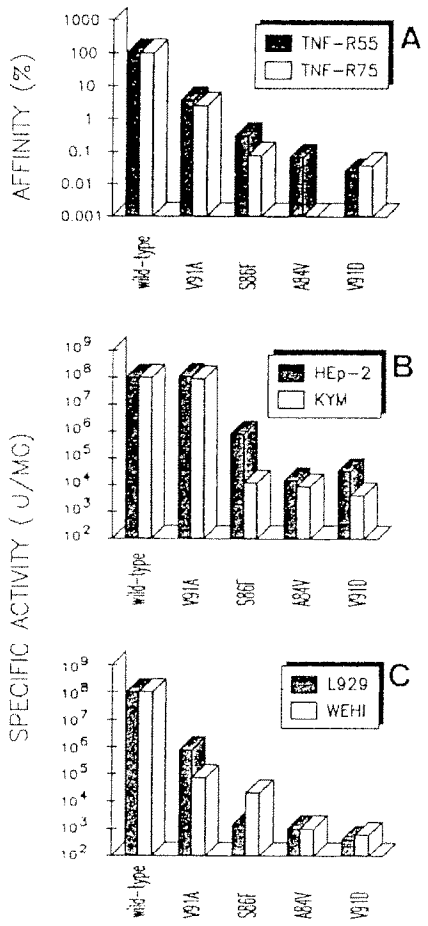


Fig. 5. Relative physical binding mediated by the two types of hTNF-R and biological activities for TNF mutants in the loop at positions 84–88 in hTNF. Further details are as indicated in the legend to Fig. 1.

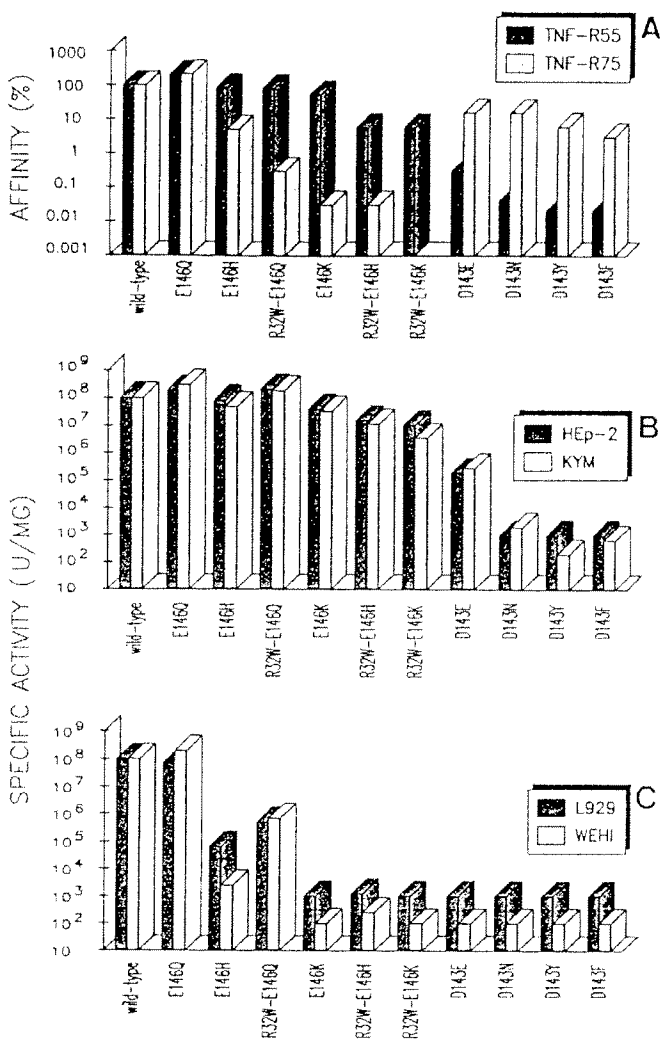


Fig. 6. Relative physical binding mediated by the two types of hTNF-R and biological activities for TNF mutants at positions 146 (without or in combination with R32W) and 143. Further details are as indicated in the legend to Fig. 1.

and largely maintained R55 affinity), the latter mutation being the most discriminative. This means that, although the affinity for hR55 was somewhat decreased for the E146K mutant (50% of wt), the reduction of hR75 binding was 5×10^3-fold, resulting in a differential binding, even more pronounced than in the case of R32W (Fig. 6; Table 1). Indeed, while R32W still had a low GM-CSF-inducing activity in hR75-transfected PC60 cells (Fig. 2), no activity of the E146K mutant could be observed in these cells (Fig. 6; Table 1). Inspection of the three-dimensional structure (Fig. 7) reveals that residue E146 is positioned near residues 29 and 32, thereby extending the region involved in the specific R75 binding towards the top of the trimeric, pyramidal TNF. In contrast, the conservative E146Q mutant did not show any differentiating effect either by binding or by biological activity. On the whole, the bioactivities of these mutants (E146Q, E146H, E146K) on HEp-2 and KYM cells, and on hR75-transfected PC60 cells, paralleled the physical binding to R55 and R75, respectively.

R55/R75 differential binding could be extended by combining the E146 and the R32W mutations. Compared with their respective single mutants at position 146, the double mutants R32W-E146H and R32W-E146K showed a reduction in affinity for both receptors. As the decrease in R55 affinity was relatively minor (15-fold), the net difference in binding affinity for both types of receptor was increased (Fig. 6; Table 1). No hR75 bioactivity was detectable even at concentrations up to 10 µg/ml (data not shown). Thus, in contrast to the double mutant L29S-R32W (Fig. 3), a cooperative effect was obtained with the combination of mutations at positions 32 and 146. Since both positions are located on different loops, mutations in these loops can induce different disruptions of contact such as local conformational rearrangements or electrostatic repulsions, that might strenghten each other's effect.

Since Yamagishi et al. (1990) reported a complete loss of cytotoxic activity and receptor binding (which receptor was not identified) with a D143Y mutant, we further investigated the role of this residue in the TNF-active site. To that end, we constructed, in addition to this non-conservative substitution, one similar (D143F) and two conservative mutations (D143E and D143N). All these mutants indeed showed a profound decrease in hR55-mediated cytotoxicity on HEp-2 and KYM cells. However, unexpectedly, these mutants retained a certain affinity for hR75 (3–15%), which was also reflected in the hR75-mediated bioactivity (10–50%, although with substantially reduced plateau levels as compared to wt hTNF; Table 1; Fig. 6). This R75 specificity was most

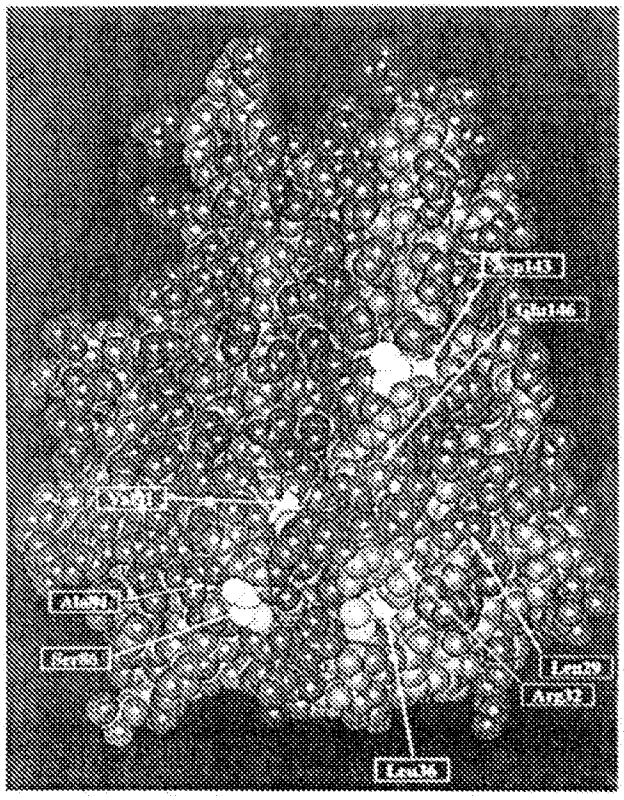


Fig. 7. Three-dimensional representation of the TNF structure with the different subunits in blue, red and yellow. The $C\alpha$-backbone of the left and right subunits in front are marked light blue and light red, respectively, while amino acid side chains are colored more intensely. Residues involved in receptor binding (Van Ostade et al., 1991) are light green; the residues found to be selectively involved in R55 (D143) or R75 (L29, R32 and E146) interaction are marked white and dark green, respectively.

pronounced with mutant D143N. It is remarkable that substitutions of two amino acids in close vicinity (D143 and E146) cause such opposite selective TNF receptor binding and bioactivity. On a three-dimensional model (Fig. 7), however, there is a clear separation between D143 and the L29-R32-E146 cluster, suggesting that in this region the two receptor-binding regions do not spatially overlap.

How the discrimination of receptor binding occurs at the molecular level, is still an open question. One may postulate structural distortions and changes in chemical bond formation in a discrete region at the site of mutation, but alternatively, one cannot rule out conformational effects at some distance from the site of mutation. Indeed, many substitutions, which resulted in a large difference in receptor binding (L29Y, R32W and E146K), were drastic as far as the chemical nature of the side chain is concerned, and thus might result in some structural changes in the neighborhood. Recently, Banner et al. (1993) described the three-dimensional structure of the lymphotoxin/R55 complex. On the basis of changes in solvent-accessible surface area after complex formation, two major binding regions could be identified on the lymphotoxin molecule. When projected on the TNF structure, the lower region encloses three loops (positions 30–60, 84–88 and 138–150), previously identified by mutagenesis studies as being involved in receptor interaction (Yamagishi et al., 1990; Goh et al., 1991; Van Ostade et al., 1991; Zhang et al., 1992). All the mutations characterized by us (which have drastically decreased receptor binding and biological activity, yet a correctly folded three-dimensional structure), are located in or near one of these loops. Concerning the upper contact region, less is known from mutagenesis studies, which can possibly be attributed to the localization of the relevant residues inside four β-strands near the top of the trimer. Modifications at these positions may cause large structural alterations, resulting in insoluble or unproperly folded proteins. Less drastic, conservative alterations might reveal to what extent this region also shows differential interaction with R55 and R75. Although the primary amino acid sequence of both receptors is fairly different, it seems that the ligand-binding site must be quite similar as shown by the concomitant reduction in binding affinity with muteins, such as A84V, S86F, V91D and many others. Obviously, the TNF receptors have at least one non-common region, involved in the specific binding, which can be analyzed in molecular detail after crystallization and subsequent X-ray diffraction studies of differentially binding muteins such as E146K and D143N.

In conclusion, this study has provided tools to investigate the distinct functions of both receptor types on different cells. R55-specific mutants such as E146K specifically stimulate R55, which is solely able to mediate the cytotoxic activity. This might allow the application of an R55-specific mutant as an antitumor agent without interference by R75-mediated events. Indeed, triggering of R75 is believed to play a major role in TNF-induced systemic toxicity (Fiers, 1993). Experiments with human neutrophils and endothelial cells have confirmed that on these the R55-specific mutant E146K has a clearly lower proinflammatory activity than wtTNF (Barbara et al., 1994).

The authors thank A. Verhee, F. Van Houtte and W. Burm for technical assistance, as well as D. Moens for protein-modeling work. They are indebted to Drs H.-R. Loetscher and W. Lesslauer (Hoffmann-La Roche) for providing binding data on TNF receptors and biological reagents.

REFERENCES

Banner, D. W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H.-J., Broger, C., Loetscher, H. & Lesslauer, W. (1993) Crystal structure of the soluble human 55 kd TNF receptor-human TNFβ complex: Implications for TNF receptor activation. *Cell 73*, 431–445.

Barbara, J. A. J., Smith, W. B., Gamble, J. R., Van Ostade, X., Vandenabeele, P., Tavernier, J., Fiers, W., Vadas, M. A. & Lopez, A. F. (1994) Dissociation of TNF-α cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor-selective TNF-α mutants, *EMBO J. 13*, in the press.

Brouckaert, P. G. G., Leroux-Roels, G. G., Guisez, Y., Tavernier, J. & Fiers, W. (1986) *In vivo* anti-tumour activity of recombinant human and murine TNF, alone and in combination with murine IFN-gamma, on a syngeneic murine melanoma. *Int. J. Cancer 38*, 763–769.

Brouckaert, P., Libert, C., Everaerdt, B. & Fiers, W. (1992) Selective species specificity of tumor necrosis factor for toxicity in the mouse, *Lymphokine Cytokine Res. 11*, 193–196.

Cerami, A. & Beutler, B. (1988) The role of cachectin/TNF in endotoxic shock and cachexia, *Immunol. Today 9*, 28–31.

Creasey, A. A., Doyle, L. V., Reynolds, M. T., Jung, T., Lin, L. S. & Vitt, C. R. (1987) Biological effects of recombinant human tumor necrosis factor and its novel muteins on tumor and normal cell lines, *Cancer Res. 47*, 145–149.

Engelmann, H., Holtmann, H., Brakebusch, C., Shemer Avni, Y., Sarov, I., Nophar, Y., Hadas, E., Leitner, O. & Wallach, D. (1990) Antibodies to a soluble form of a tumor necrosis factor (TNF)

receptor have TNF-like activity, *J. Biol. Chem. 265*, 14497–14504.

Espevik, T. & Nissen-Meyer, J. (1986) A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes, *J. Immunol. Methods 95*, 99–105.

Espevik, T., Brockhaus, M., Loetscher, H., Nonstad, U. & Shalaby, R. (1990) Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor, *J. Exp. Med. 171*, 415–426.

Fiers, W. (1993) Tumour necrosis factor, in *The natural immune system: humoral factors* (Sim, E., ed.) pp. 65–119, IRL Press, Oxford.

Fransen, L., Van der Heyden, J., Ruysschaert, R. & Fiers, W. (1986) Recombinant tumor necrosis factor: Its effect and its synergism with interferon-γ on a variety of normal and transformed human cell lines, *Eur. J. Cancer Clin. Oncol. 22*, 419–426.

Goh, C. R., Loh, C.-S. & Porter, A. G. (1991) Aspartic acid 50 and tyrosine 108 are essential for receptor binding and cytotoxic activity of tumour necrosis factor beta (lymphotoxin), *Protein Eng. 4*, 785–791.

Hohmann, H.-P., Brockhaus, M., Baeuerle, P. A., Remy, R., Kolbeck, R. & Van Loon, A. P. G. M. (1990) Expression of the types A and B tumor necrosis factor (TNF) receptors is independently regulated, and both receptors mediate activation of the transcription factor NF-κB. TNFα is not needed for induction of a biological effect via TNF receptors, *J. Biol. Chem. 265*, 22409–22417.

Lesslauer, W., Tabuchi, H., Gentz, R., Brockhaus, M., Schlaeger, E. J., Grau, G., Piguet, P. F., Pointaire, P., Vassalli, P. & Loetscher, H. (1991) Recombinant soluble tumor necrosis factor receptor proteins protect mice from lipopolysaccharide-induced lethality, *Eur. J. Immunol. 21*, 2883–2886.

Lewis, M., Tartaglia, L. A., Lee, A., Bennett, G. L., Rice, G. C., Wong, G. H. W., Chen, E. Y. & Goeddel, D. V. (1991) Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific, *Proc. Natl Acad. Sci. USA 88*, 2830–2834.

Loetscher, H., Pan, Y. E., Lahm, H. W., Gentz, R., Brockhaus, M., Tabuchi, H. & Lesslauer, W. (1990) Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor, *Cell 61*, 351–359.

Loetscher, H., Gentz, R., Zulauf, M., Lustig, A., Tabuchi, H., Schlaeger, E.-J., Brockhaus, M., Gallati, H., Manneberg, M. & Lesslauer, W. (1991) Recombinant 55-kDa tumor necrosis factor (TNF) receptor. Stoichiometry of binding to TNFα and TNFβ and inhibition of TNF activity, *J. Biol. Chem. 266*, 18324–18329.

Meager, A. (1991) A cytotoxicity assay for tumour necrosis factor using a human rhabdomyosarcoma cell line, *J. Immunol. Methods 114*, 141–143.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *J. Immunol. Methods 65*, 55–63.

Nakamura, S., Kato, A., Masegi, T., Fukuoka, M., Kitai, K., Ogawa, H., Ichikawa, Y., Maeda, M., Watanabe, N., Kohgo, Y. & Niitsu, Y. (1991a) A novel recombinant tumor necrosis factor-α mutant with increased anti-tumor activity and lower toxicity, *Int. J. Cancer 48*, 744–748.

Nakamura, S., Masegi, T., Fukuoka, M., Kitai, K., Kato, A., Ichikawa, Y., Watanabe, N. & Niitsu, Y. (1991b) Interactions between novel tumor necrosis factor-α mutants and receptors on tumor and normal cells, *Agric. Biol. Chem. 55*, 53–57.

Pennica, D., Kohr, W. J., Fendly, B. M., Shire, S. J., Raab, H. E., Borchardt, P. E., Lewis, M. & Goeddel, D. V. (1992) Characterization of a recombinant extracellular domain of the type 1 tumor necrosis factor receptor: Evidence for tumor necrosis factor-α induced receptor aggregation, *Biochemistry 31*, 1134–1141.

Ranges, G. E., Bombara, M. P., Aiyer, R. A., Rice, G. G. & Palladino, M. A. Jr (1989) Tumor necrosis factor-α as a proliferative signal for an IL-2-dependent T cell line: Strict species specificity of action, *J. Immunol. 142*, 1203–1208.

Ruff, M. R. & Gifford, G. E. (1981) Tumor necrosis factor, in *Lymphokines* (Pick, E., ed.) vol. 2, pp. 235–272, Academic Press, New York.

Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H. W., Gatanaga, T., Granger, G. A., Lentz, R., Raab, H., Kohr, W. J. & Goeddel, D. V. (1990) Molecular cloning and expression of a receptor for human tumor necrosis factor, *Cell 61*, 361–370.

Shalaby, M. R., Sundan, A., Loetscher, H., Brockhaus, M., Lesslauer, W. & Espevik, T. (1990) Binding and regulation of cellular functions by monoclonal antibodies against human tumor necrosis factor receptors, *J. Exp. Med. 172*, 1517–1520.

Sidhu, R. S. & Bollon, A. P. (1989) Tumor necrosis factor analogs: Identification of functional domains, *Anticancer Res. 9*, 1569–1576.

Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D. & Goodwin, R. G. (1990) A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins, *Science 248*, 1019–1023.

Sprang, S. R. & Eck, M. J. (1992) The 3-D structure of TNF, in *Tumor necrosis factors: the molecules and their emerging role in medicine* (Beutler, B., ed.) pp. 11–32, Raven Press, New York.

Spriggs, D. R. & Yates, S. W. (1992) Clinical studies of tumor necrosis factor in the USA, in *Tumor necrosis factor: structure-function relationship and clinical application* (Osawa, T. & Bonavida, B., eds) pp. 275–284, Karger, Basel.

Stanssens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M. & Fritz, H.-J. (1989) Efficient oligonucleotide-directed construction of mutations in expression vectors by the gapped duplex DNA method using alternating selectable markers, *Nucleic Acids Res. 17*, 4441–4453.

Tartaglia, L. A., Weber, R. F., Figari, I. S., Reynolds, C., Palladino, M. A. Jr & Goeddel, D. V. (1991) The two different receptors for tumor necrosis factor mediate distinct cellular responses, *Proc. Natl Acad. Sci. USA 88*, 9292–9296.

Tartaglia, L. A. & Goeddel, D. V. (1992) Two TNF receptors, *Immunol. Today 13*, 151–153.

Tartaglia, L. A., Pennica, D. & Goeddel, D. V. (1993) Ligand passing: The 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor, *J. Biol. Chem. 268*, 18542–18548.

Tavernier, J., Marmenout, A., Bauden, R., Hauquier, G., Van Ostade, X. & Fiers, W. (1990) Analysis of the structure-function relationship of tumour necrosis factor. Human/mouse chimeric TNF proteins: General properties and epitope analysis, *J. Mol. Biol. 211*, 493–501.

Vandenabeele, P., Guisez, Y., Declercq, W., Bauw, G., Vandekerckhove, J. & Fiers, W. (1990) Response of murine cell lines to an IL-1/IL-2-induced factor in a rat/mouse T hybridoma (PC60): Differential induction of cytokines by human IL-1α and IL-1β and partial amino acid sequence of rat GM-CSF, *Lymphokine Res. 9*, 381–389.

Vandenabeele, P., Declercq, W., Vercammen, D., Van de Craen, M., Grooten, J., Loetscher, H., Brockhaus, M., Lesslauer, W. & Fiers, W. (1992) Functional characterization of the human tumor necrosis factor receptor p75 in a transfected rat/mouse T cell hybridoma, *J. Exp. Med. 176*, 1015–1024.

Van Ostade, X., Tavernier, J., Prangé, T. & Fiers, W. (1991) Localization of the active site of human tumour necrosis factor (hTNF) by mutational analysis, *EMBO J. 10*, 827–836.

Van Ostade, X., Vandenabeele, P., Everaerdt, B., Loetscher, H., Gentz, R., Brockhaus, M., Lesslauer, W., Tavernier, J., Broeckaert, P. & Fiers, W. (1993) Human TNF mutants with selective activity on the p55 receptor, *Nature 361*, 266–269.

Yamagishi, J., Kawashima, H., Matsuo, N., Ohue, M., Yamayoshi, M., Fukui, T., Kotani, H., Furuta, R., Nakano, K. & Yamada, M. (1990) Mutational analysis of structure-activity relationships in human tumor necrosis factor-alpha, *Protein Eng. 3*, 713–719.

Zhang, X.-M., Weber, I. & Chen, M.-J. (1992) Site-directed mutational analysis of human tumor necrosis factor-α receptor-binding site and structure-functional relationship, *J. Biol. Chem. 267*, 24069–24075.

Vol. 38, No. 6, May 1996 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 1183–1189

BIOLOGICAL ACTIVITIES OF HUMAN TUMOR NECROSIS FACTOR-ALPHA AND ITS NOVEL MUTANTS

Tao Xi, Xiaoqing Shi, Donglin Guo, Xueyin Dong, Xianxiu Xu, Dexu Zhu*

Department of Biochemistry and State Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, People's Republic of China

Kiaki Han

L'Unité N°377 l'INSERM, Place de Verdum, 59045 Lille Cédex, France [Tel. 0033-20-29-88-50; Fax 0033-20-53-85-62 (INSERM-CHR-LILLE)]

Received February 7, 1996

Summary: Biological activities of human tumor necrosis factor-alpha (hTNF-α) and its mutants were compared. In cytotoxicity assay with L929 cells, one mutant, designated as TNF-B, showed 4.5-fold higher activity than TNF examined. In receptor binding assay, TNF-B had almost the same affinity for TNF receptors on L929 cells as hTNF-α. We also found that TNF-B retained the cytotoxicity of hTNF-α for HEp-2 cells. TNF-B also had two-fold higher affinity than hTNF-α for receptors on HEp-2 cells (only carrying hTNF-R55) and lower affinity for receptors on U937 cells (expressing mainly hTNF-R75). These results suggested that TNF-B might still interact with the human TNF-R55 receptor, but it might largely lose its ability to bind to human TNF-R75. Changes of biological activity of TNFs might be due to an altered affinity to the different types of TNF receptor on the target cells.

Tumor necrosis factor-alpha (TNF-α), a 17 kD protein mainly produced by activated monocytes/macrophages was originally described as a mediator of lipopolysaccharide-induced haemorrhagic necrosis of certain murine tumors and a molecule with cytostatic/cytotoxic activity on tumor cells in culture(1,2). It has now been shown to mediate a wide variety of biological responses both in vivo and in vitro(3). The active form of hTNF-α is believed to be a trimer (4,5). Each monomer contains 157 amino acid residues. The crystal structure of hTNF-α has been determined(6). TNF mediates its activities by binding to specific high affinity receptors on the surface of cells (7,8); the majority of mammalian cells carry receptors for TNF (9). Two distinct TNF receptors of approximately 55kD (TNF-R55) and 75 kD(TNF-R75) have now been identified (8,10). Since TNF induces such a wide spectrum of activities, it is likely that some of these responses are mediated by TNF-R55, others by TNF-R75. TNF has been shown to mediate both cell proliferation and cell death depending on the cell type or culture conditions. Two recent studies have implicated specific receptors in mediating cellular

* To whom correspondence should be addressed.

1039–9712/96/061183–07$05.00/0

EXHIBIT D

cytotoxicity but with conflicting results (11, 12). In this report, we prepared two novel hTNF-α mutants to investigate their binding to two types of TNF receptors and their biological activities. We also investigated whether the changes of biological activities were due to an altered affinity to the different types of receptors on the target cells.

MATERIALS AND METHODS

Materials : human recombinant TNF-α was produced in *E.coli* by using a pET11d plasmid. The genes of hTNF-α mutants were obtained by in vitro site-specific mutagenesis based on the PCR technique (13). Figure 1 showed the primary structures of hTNF-α and its mutants. A mutant was designated as TNF-B which amino terminal 3-8 amino acid residues (Ser-Ser -Ser-Arg-Thr-Pro) were replaced with two amino acid residues (Lys-Arg). Another mutant was named as TNF-arg with the single proline 20 substituted by arginine. TNFs had a purity of ≥ 99% as shown by SDS-PAGE and they were immunoblotted with polyclonal anti-hTNF-α antibodies.

Cell lines and culture: the L929 mouse fibroblast cell line, the human larynx carcinoma-derived cell line HEp-2 and the human monoblastoid leukaemic cell line U937 were obtained from the American Type Culture Collection. L929 cells were cultured in Eagle's minimum essential medium with 10% neonatal calf serum, 100IU/ml penicillin and 100μg/ml streptomycin. U937 and HEp-2 cells were grown in RPMI-1640 with 10% fetal calf serum, 100IU/ml penicillin, 100μg/ml streptomycin, and 0.3 mg/ml L-glutamin at 37°C , 5% CO_2 in a CO_2 incubator.

Cytotoxicity against murine L929 fibroblast cell line : the specific activity of hTNF-α and its mutants were determined with L929 cells (2). 3×10^4 cells /well were seeded into 96-well microtiter plates in 100 μl of culture medium and incubated at 37°C, 5% CO_2. After 20 hr, adherent cells were incubated in meduim containing ten fold serial dilutions of TNF and 1 μg/ml actinomycin D at 37°C for another 20 hr. Dead cells were removed by washing. Viable cells were stained with 0.5% crystal violet for 20 min, washed with water , and solubilized in 33% acetic acid. The absorbance at 595 nm was measured.

Inhibition of DNA and protein synthesis of human monoblastoid leukaemic cell line U937: U937 cells (1×10^5 cells/well) were incubated in 96-well microtiter plates in RPMI 1640 medium supplemented with 10% fetal calf serum and a serial dilutions of hTNF-α and its mutants at 37°C , 5% CO_2 for 3 days. To determine the cell proliferation, 37 MBq/L [^{3}H]

	1 2 3 4 5 6 7 8	20	155 156 157
hTNF-α	Val-Arg-Ser- Ser-Ser-Arg-Thr-Pro-	-Pro-	-Ile-Ala-Leu
TNF-B	Val-Arg-Lys-Arg-	-Pro-	-Ile-Ala-Leu
TNFarg	Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-	-Arg-	-Ile-Ala-Leu

Figure 1: Sequences of tested hTNF-α mutants compared with the sequence of the native human TNF-α molecule.

thymidine (26 mCi/mmol; 1 Ci=37 GBq; New England Nuclear) was added to each well. Cells were incubated for 16 hr and harvested onto glass fiber filters. [^{3}H] thymidine incorporation (cpm) of triplicate cultures was determined by using a liquid scintillation counter (Packard 1600 TR). For analysis of protein synthesis , the same procedure was followed with 37 MBq/L [^{3}H] leucine (2.04 PBq/mol, Shanghai Nucleal Institute).

Radioiodination of human TNF: human TNF-α was iodinated with ^{125}I by the iodogen method (14). Briefly, 20μg TNF-α and 2 mCi of Na^{125}I in 100μl PBS were incubated with a glass bead coated with 0.47 μg iodogen for 1 min . The labeled TNF-α was separated from free iodine by filtration on a Sephadex G 25 column equilibrated with PBS and saturated with 0.2%BSA. The [^{125}I] TNF had a specific activity of 0.4 MBq/ g. The majority (80%) of the bioactivity was retained , as determined by the L929 cytotoxicity assay.

Receptor binding assay: the binding assays were performed by using the 24-well method . Briefly, the target cells were seeded in 24-well tissue culture plates at 2.5 $\times 10^5$ cells/well and incubated overnight at 37 °C, 5%CO_2 . For Scatchard analysis, a serial dilution (6.4nM to 0.024nM) of the labelled hTNF-α was prepared in triplicate wells. Nonspecific binding was determined by adding 200-fold excess of unlabelled hTNF-α to each well. For the competitive radiolabelled ligand assay , a serial dilution (100 nM to 0.1 nM) of unlabelled TNFs and 0.6 nM [^{125}I] TNF-α were added to triplicate wells. The two remained wells contained only labelled hTNF-α (total binding), and a 500-fold excess of unlabelled hTNF-α (background), respecitively. The all reactions were done in 0.5 ml cell culture medium at 4 °C for 2 hr. Unbound [^{125}I] TNF-α was removed by washing with cold PBS and the radioactivity bound to the cells was quantified in a gamma counter. The relative receptor binding inhibition activity (RBI) of hTNF-α munant is defined as followed :

RBI of hTNF-α mutant = (ID50 hTNF-α /ID50 hTNF-α mutant)x 100%

ID50, half-inhibition dose, is defined as the amount of a protein to inhibit 50% of the [^{125}I] TNF-α binding in the receptor binding inhibition assay.

RESULTS

***in vitro* cytotoxicity against L929 cells and HEp-2 cells:** in the experiments, we compared the cytotoxic activity of hTNF-α and its mutants against murine L929 fibroblasts and human larynx carcinoma-derived cell line HEp-2. As shown in Table 1 , hTNF-α had a specific activity of 2×10^7 U/mg protein against L929 cells. Compared with hTNF-α , the specific activity of TNF-B was increased by 4.5-fold, but the specific activity of TNFarg was decreased to 0.1% of that of the control hTNF-α . We also found that HEp-2 cells was susceptible to the cytotoxic action of hTNF-α in the presence of cycloheximinde. TNF-B showed about 2-fold higher activity than hTNF-α , but TNFarg showed much lower activity than hTNF-α . These results suggested that the cytotoxicity of TNFs to tumor cells might be nonspecies specific.

Inhibition of DNA and protein synthesis of U937 cells: the human monoblastoid leukaemic cell line U937 1×10^5 cells/well were incubated with various concentrations of hTNF- α or its mutants at 37 °C for 3 days. As show in Figure 2 , the [^{3}H]thymidine and [^{3}H]leucine incorporations of U937 cells decreased faster in the presence of TNF-B than hTNF-α .So the

Table 1 Comparison of the cytotoxic activities of human TNF and its mutants on L929 and HEp-2 cells. L929 cells were seeded in 96-well plates, and incubated with various concentration of TNF and 1 ug/ml actinomycin D for 20 hr. The same procedure was used for HEp-2 cells, except that 50 ug/ml cycloheximide was added instead of actinomycin D. Specific activity was determined as described in " MATERIALS AND METHODS ". The values represent the mean±SD of eight experiments and relative values (taking TNF as 100%) are indicated in brackets.

TNFs	L929 Specific activity (U/mg)	HEp-2 Specific activity (U/mg)
TNF	$2(\pm 0.4)\times 10^{7}$ (100%)	$1.5(\pm 0.6)\times 10^{7}$ (100%)
TNF-B	$9(\pm 3.4)\times 10^{7}$ (450%)	$2.8(\pm 0.8)\times 10^{7}$ (187%)
TNF-arg	$2(\pm 0.2)\times 10^{4}$ (0.1%)	$6.6(\pm 1.2)\times 10^{3}$ (0.04%)

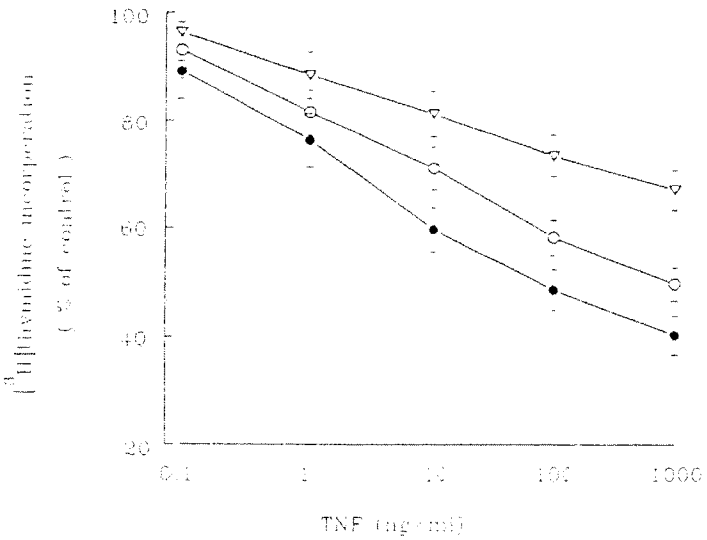


Figure 2: Cytostatic activity of human TNF-α mutants against the human monoblastic leukaemia cell line U937. Leukaemia cells were incubated with the TNFs for 3 days. To determine proliferation, cells were pulsed with [^{3}H]thymidine for 16 hr. Values of [^{3}H]thymidine incorporation are related to the medium control and represent the mean ±SD of three experiments. (○)hTNF-α; (●) TNF-B; (Δ) TNF-arg.

inhibition of TNF-B to the DNA and protein synthesis of U937 cells was higner than that of TNF-α . But the inhibition of TNFarg was lower than that of TNF-α (Fig. 3). The differences of inhibition activity between TNF-α and its mutants may be due to the differences of cytotoxicity between them.

Competitive radiolabelled ligand binding assay: in order to determine whether the altered biological activities of TNF-B and TNFarg were caused by altered affinities to the target cells , competitive radiolabelled ligand binding assays were preformed. It was shown that the hTNF- α mutants differed in their ability to compete with ^{125}I-labelled hTNF-α for binding to the target cells (Table 2). Used as a competitive inhibitor for [^{125}I]TNF-α binding to L929 cells, TNF-B almost had the same inhibition activity compared to hTNF-α. But the concentration of TNFarg required to inhibit 50% of the binding was 40-fold higher than that of hTNF-α. We also tried on HEp-2 and U937 cell lines in this experiment and found that the concentration of TNF-B required to 50% of binding to HEp-2 cells was about 2-fold lower and 40-fold higher to U937 than that of hTNF-α , respectively. The affinity of TNFarg for the receptors was much lower than that of hTNF-α on both target cells.

DISCUSSION

Previous experiments with TNF-deletion mutants have shown that the extensive changes of the amino-terminus with deletions of as many as eight amino acid residues do not reduce the cytotoxicity significantly (15). In fact, the removal of up to eight amino-terminal amino acid residues even increases two to three fold of the activity (16). However,the deletion of more than ten amino-teminal amino acid residues decreases the specific activity (17), and some amino acid residues among 11-31 are important for maintaining the structure and cytotoxic activity . These results might be caused by three-dimensional structure of TNF. Since the receptors binding site might localize in the bottom of TNF-α , the deletion of the amino-terminal residues proximity to Lys-11 may disturb the local conformation which can indirectly influence the activity. The mutant TNF-B has the 3-8 amino acid residues Ser-Ser- Ser- Arg-Thr-Pro replaced with two basic amino acid residues Lys-Arg. Its specific activity against L929 cells increased 4.5-fold , which is higher than that of the mutant with the deletion of only these amino terminal residues. TNF-B might maintain a more favorable conformation for bioactivity at the bottom of the trimer. Another mutant, designated as TNFarg with the single proline-20 substituted by argnine, markedly decreased the overall activity of hTNF-α for different types of target cell. These results suggested that Pro-20 might be very important for maintaining the structure and the biological activity of TNF.

In this report , the competitive inhibition experiments on three cell lines showed that TNF-B had the similar affinity to L929 cells compared to TNF-α and its affinity to HEp-2 cells was

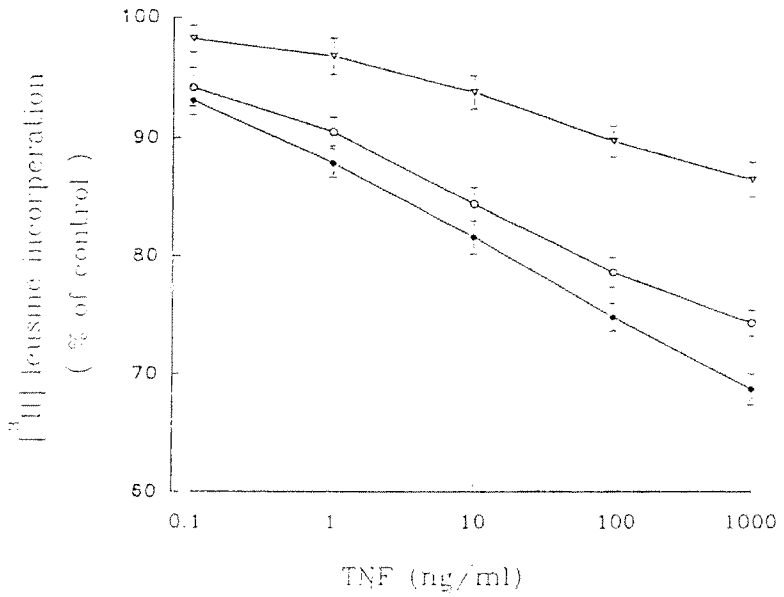


Figure 3: Effects of human TNF-α mutants on [^{3}H]leucine incorporation in cultured human monoblastic leukaemia cell line U937. Leukaemia cells were incubated with the TNFs for 3 days. To determine protein synthesis, cells were pulsed with [^{3}H]leucine for 16 hr. Values of [^{3}H]leucine incorporation are related to the medium control and represent the mean±SD of three experiments. (○)hTNF-α; (●) TNF-B; (△) TNF-arg.

Table 2. Affinity of human TNF-α and its mutants on L929, HEp-2 and U937 cells. Binding of hTNF-α on three target cells was determined by Scatchard analysis. K_D values of hTNF-α mutants were found by competition analysis. RBI values (taking TNF as 100%) are indicated in brackets. All experimental procedures were described in " MATERIALS AND METHODS ".

	L929		HEp-2		U937	
	Binding K_D	ID_{50} nM	Binding K_D	ID_{50} nM	Binding K_D	ID_{50} nM
TNF	1.2×10^{-10}	1.06 (100%)	5.2×10^{-10}	3.23 (100%)	3.4×10^{-8}	2.47 (100%)
TNF-B	1.3×10^{-10}	1.14 (93%)	2.6×10^{-10}	1.62 (199%)	5.5×10^{-9}	98.9 (6.2%)
TNF-arg	5.0×10^{-9}	43.8 (2.4%)	7.7×10^{-9}	47.8 (6.7%)	1.0×10^{-8}	74.6 (3.3%)

higher than that of TNF-α . But the experiments also showed that the affinity of TNF-B to U937 cells was much lower than that of TNF-α . It was reported that TNF had two different receptors (12) . HEp-2, a human larynx carcinoma-derived cell line, only carries hTNF-R55. U937 , a human monoblastoid leukaemic cell line , expresses mainly hTNF-R75 and also some hTNF-R55 (7) . hTNF has about 5-fold higher affinity to TNF-R75 than to TNF-R55(22). Our results indicated that TNF-B largely lost its ability to bind hTNF-R75, but it still retained its binding capacity to TNF-R55. Hence, we believe that TNF-R55 may be implicated as a major mediator of TNF signalling and the activation of hTNF-R55 may be sufficient to trigger cytotoxic activity towords transformed cells.

REFERENCES

1. Carswell E.A., Old L.J., Kassel R.L., Green S., Fiore N. and Williamson B. (1975) Proc.Natl.. Acad. Sci. U.S.A. 72 ,3666-3670.
2. Creasey A.A., Doyle L.V., Reynolds M.T., Jung T., Lin L.S.and Vitt C.R. (1987) Carcer Res. 47, 145-149.
3. Kircheis R., Milleck J., Korobko V.G., Shingarova L.N., Behnke D. and Schmidt H.E. (1992) Immunology 76, 433-438.
4. Kunitani M.G., Cunico R.L. and Stats S.T. (1988) J. Chromatogr. 433, 205-220.
5. Smith R.A. and Baglioni C. (1987) J. Biol. Chem. 262, 6951-6954.
6. Jones E.Y., Stuart D.I.and Walker N.P.C.(1989) Nature 338, 225-228.
7. Brockhaus M., Schoenfeld H. J. Schlaeger E. J.,Hunkziker W., Lesslauer W. and Loetscher H.. (1990) Proc. Nalt. Acad. Sci. U.S.A. 87, 3217-3131.
8. Hohmann H.P., Remy R., Brockhaus M. and van Loon A.P.G.M. (1989) J.Biol. Chem. 264, 14927-14934.
9. Aggarwal B.B., Eessalu T. E. and Haas P.E. (1985) Nature 318, 665-667.
10. Engelmann H., Novick D. and Wallach D.(1990) J. Biol. Chem. 265, 1531-1536.
11. Heller R.A., Song K.,Fan N. and Chang D.J. (1992) Cell 70, 47-56.
12. Tartaglia L.A , Weber R.F., Figari I.S., Reynolds C., Palladino M.A., Jr and Goeddel D.V. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 9292-9296.
13. Gase K., Korobko V.G., wisniewski H.G , Le J., Dobrynin V.N.,Filippov S.A., Gutsche W., Maksimova Y.N., Schlott B., Shingarova L.N., Vilcek J. and Beknke D. (1990) Immunology 71,368-371.
14. Markwell M.A.K. (1982) Anal. Biochem. 125, 427-432.
15. Carlino J.A., Lin L.S.and Creasey A.A. (1987) J. Biol. Chem. 262, 958-961.
16. Sidhu R.S. and Bollon A.P. (1989) Anticancer Res. 9 , 1569-1576
17. Nakamura S., Masegi T., Fukuoka M., Kitai K., Kato A., Ichikawa Y., Watanabe N. and Niitsu Y. (1991) Agricult. Biol. Chem. 55, 53-58.
18. Tartaglia L.A. and Goeddel D. V. (1992) Immun. Today 13, 151-153.

Vol. 44, No. 6, May 1998 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 1075–1082

A NOVEL TUMOR NECROSIS FACTOR-α MUTANT WITH SIGNIFICANTLY ENHANCED CYTOTOXICITY AND RECEPTOR BINDING AFFINITY

Nam-Kyu Shin, Inkyoung Lee, Seung-Gu Chang and Hang-Cheol Shin*

Protein Engineering Laboratory, Hanhyo Institute of Technology
461-6 Jeonmin-dong, Yusong-ku, Taejon #305-390, Republic of Korea

Received December 19, 1997

SUMMARY

A novel tumor necrosis factor-α mutant (mutant M3), in which Ser and Tyr at positions 52 and 56 were substituted by Ile and Phe, respectively, along with deletion of 7 N-terminal amino acids, was prepared and its biological activities were investigated. The mutant exhibited a 14- to 24-fold increase in the cytotoxicity relative to the wild-type TNF on various cancer cell lines. The binding affinity of the mutant to TNF-R55 and TNF-R75 receptors was over 10-fold higher than that of the wild-type. TNF-α and the mutant show similar CD spectra in the far-UV region, indicating that the overall structure was not influenced by the mutations. The production of highly potent TNF-α mutant utilizing increase of hydrophobicity in the region 52-56 may provide a structural basis for a design of optimized TNF-α as a therapeutic purpose.

INTRODUCTION

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine with a wide range of biological activities, including antitumor activity, immune cell proliferation and mediation of inflammatory responses (1,2). Its *in vivo* antitumor effects are either direct, resulting from cytotoxic action on tumor cells, or indirect, through the modulation of antitumor immune rejection responses or damage to tumor vasculature. The clinical use of TNF-α as an antitumor agent, however, has been limited by the proinflammatory side-effects including hypotension, hepatotoxicity, intravascular thrombosis and haemorrhage (3-6). Thus designing clinically applicable TNF-α mutants with improved therapeutic index has been the subject of considerable pharmacological interest (6-8). TNF has also shown potential for use in combination with other anticancer agents (9).

*Corresponding author. FAX: (82) (42) 866-9129. E-mail: hcshin@donald.hanhyo.co.kr

1039–9712/98/061075–08$05.00/0

EXHIBIT E

TNF activities are mediated through two different receptors, TNF-R55 (55-kDa) and TNF-R75 (75-kDa), on the cell surface. Extracellular domains of both receptors show a significant sequence similarity with a 6-cysteine consensus motif repeated four times. On the other hand, intracellular domains do not display any obvious sequence similarity to each other, providing distinct functions for the two TNF receptors (10,11).

Several regions of TNF-α were reported to be important in the biological activities and receptor binding affinities (12-15). These regions were located in the lower half of the molecule, mainly at each side of the groove that separates two monomers in the trimeric structure. Bioactivity of TNF-α increases when deletions or substitutions were made at the amino- or carboxy-terminus, providing some structural basis in the design of clinically applicable mutant forms (15-17). Indeed, most of the TNF-α mutants having developed for preclinical studies contain modifications in these regions (18-20). Recently, N-terminal modified form of TNF-β, a sister cytokine of TNF-α, has been also reported to show improved therapeutic potential (21).

In the present study, we report a hyperactive TNF-α mutant (mutant M3), in which Ser and Tyr at positions 52 and 56 were substituted by Ile and Phe, respectively, along with deletion of 7 N-terminal amino acids. This mutant exhibits significantly enhanced bioactivity and receptor binding affinity to both of the TNF receptors. The highly potent nature of the mutant may provide another and yet significantly important structural basis in the design of anticancer therapeutics of TNF-α.

MATERIALS AND METHODS

Preparation of recombinant TNFs. Human TNF-α gene was prepared through polymerase chain reaction (PCR) using Lambda gt11 U937 human monocyte cDNA library as template DNA. The gene was cloned into plasmid pT7-7 containing T7 promoter system and the resulting plasmid pT7-TNF was introduced into *E. coli* strain BL21 (DE3) as described by Hanahan (22). The gene encoding human TNF-α mutant was prepared by site-directed mutagenesis using the plasmid pT7-TNF as template and cloned into plasmid pT7-7 by the same procedure as mentioned above. *E. coli* BL21 (DE3) transformants harboring these plasmids were induced to express high levels of these proteins using standard fermentation procedures. Wild-type TNF was purified from the supernatant using a MONO-Q anion exchange chromatography. For the mutant which was produced as inclusion bodies, purification in denaturing conditions, followed by refolding with 30-fold slow dilution steps were added before MONO-Q column purification. The purity of both TNFs was above 95% as judged by SDS-PAGE analysis. *Cytotoxicity assay.* The cytotoxic activities of TNF-α and its mutant were measured on actinomycin-D treated murine L929 cells (ATCC CCL-929) as described (15), with

some modifications. Briefly, L929 cells were seeded at 1 x 10^4 cells/well into 96-well microtiter plate in Dulbecco's modified medium containing 2% fetal calf serum. 18 hours later, medium containing 2μg/ml actinomycin-D was added to the cells together with various concentrations of TNF. The cells were incubated for an additional 18 hours at 37°C. Cell viability was determined by measuring the cellular metabolic activity with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (23). The cytotoxic activities of TNF-α and its mutant on other cancer cell lines were assayed as described (24).

Receptor binding assay. Wt-TNF was iodinated with $Na^{125}I$ using Iodogen (Pierce) as oxidant. A specific radioactivity of radiolabeled TNF was 38 μCi/μg. Receptor binding experiment was carried out by the method of Ostade et al. (6) with minor modification. Labeled TNF (50 ng/ml) with various concentrations of unlabeled TNF (2-1000 ng/ml) were added to 4 x 10^6 cells for HEp-2 and 1 x 10^6 cells for U937 in 0.4ml cell culture medium supplemented with 0.1% NaN_3. After incubation for 4 hours at 4°C, cells were then washed four times with ice-cold fresh medium and lysed using lysis buffer (1% Triton X-100, 10% glycerol, 20mM Hepes, pH 7.4) to count the cell-associated radioactivity.

Circular dichroism. Circular dichroism (CD) spectra were recorded with a JASCO model J-710 spectropolarimeter at room temperature. The instrument was calibrated with androsterone/dioxane solution. Samples were prepared in quartz cells with optical pathlengths of 0.1 cm. The reported spectra are the average of several scans. Concentrations of samples were usually 0.2 mg/ml. The mean residue ellipticity, in degree.cm^2/dmol, was calculated from the relationship $[\Theta] = \theta obs \times MRW/10lc$, where θobs is the observed ellipticity, l is the pathlength in cm and c is concentration in g/ml. The mean residue weight (MRW) was calculated from the known primary sequence of the protein.

RESULTS AND DISCUSSION

The cytotoxic activities of purified TNF-α and M3 mutant were measured on various cancer cell lines, including human BT-20 breast adenocarcinoma and ME-180 cervix carcinoma cancer cell lines (Table 1). M3 mutant exhibits a 14-24 fold increase in cytotoxicity relative to the wild-type on the various cancer cell lines (BT-20, SK-BR-3, ME-180, L929, Meth-A and MH-134). This indicates that M3 mutant shows a broader cytotoxicity and no specificity among the cancer cell lines studied.

In order to compare the relative binding affinity of wt-TNF and M3 mutant to the R55 and R75 receptors, two different cell types were selected. HEp-2, a human larynx carcinoma-derived cell line, only carries TNF-R55 (25) and U937, a human promyelocytic cell line, expresses mainly TNF-R75 and also some TNF-R55 (25). As TNF-α binds with about 5-fold higher affinity to the larger receptor (11,26), suboptimal concentration of ^{125}I-wtTNF are predominantly bound to TNF-R75. Wt-TNF dose-dependently competes with labeled TNF-α for binding to TNF receptors on HEp-2 and

Table 1. *In vitro* cytotoxic activity of wild-type hTNF and M3 mutant (S52I+Y56F(Δ7)) on various cancer cell lines.

cells	specific activity (units/mg)		relative activity
	wt-TNF	M3	
human			
BT-20 (breast adenocarcinoma)	1.31×10^5	2.53×10^6	19.3
SK-BR-3 (breast adenocarcinoma)	3.00×10^2	5.34×10^3	17.8
ME180 (cervix carcinoma)	1.10×10^5	1.54×10^6	14.0
murine			
L929 (fibrosarcoma)	7.14×10^7	1.52×10^9	21.3
Meth-A (fibrosarcoma)	3.87×10^4	9.30×10^5	24.0
MH-134 (hepatoma)	4.12×10^4	7.08×10^5	17.2

U937 cells (6). As shown in Table 2, the concentration of M3 required for 50% binding inhibition of labeled-TNF to the receptors on both cells was over ten-fold lower than that of wt-TNF. This result indicates that the affinity to both R55 and R75 receptors of M3 mutant is over 10-fold higher than that of wt-TNF. The magnitude of enhanced cytotoxicity and receptor binding affinity shown by this mutant is highest amongst TNF mutants so far reported.

To examine whether the mutation causes any conformational change to TNF, the structures of wild-type and M3 mutant were compared by circular dichroism (CD). As shown in Fig. 1, the CD spectrum of TNF in water displays a typical circular dichroic pattern for a compact globular protein with high percentage of β-structure. The two proteins show similar CD spectra in the far-UV region, indicating that the double mutations introduced at positions 52 and 56 exert only minor influence on the conformation of TNF-α.

Fig. 2 shows the bottom view of the TNF molecule indicating the positions where mutations were made. According to the x-ray crystal structure of TNF-α, Ser-52 is exposed to the solvent environment and lies near Tyr-56 which is buried in the hydrophobic pocket. These positions are part of a loop structure located in the base of the bell-shaped TNF trimer (27,28). This trimeric structure was reported to be the biologically active form of TNF (29). Tyr-56 is in contact with Ile-154, Val-50, Leu-126

Table 2. Receptor binding potencies of wt-TNF and M3 mutant

TNF	potency relative to wt-TNF*	
	HEp-2	U937
wt	100	100
M3	1110	1250

*Relative receptor-binding potency is defined as ((concentration of wt-TNF causing 50% inhibition of binding of ^{125}I-wtTNF to receptor)/(concentration of M3 mutant causing 50% inhibition of binding of ^{125}I-wtTNF to receptor)) x 100.

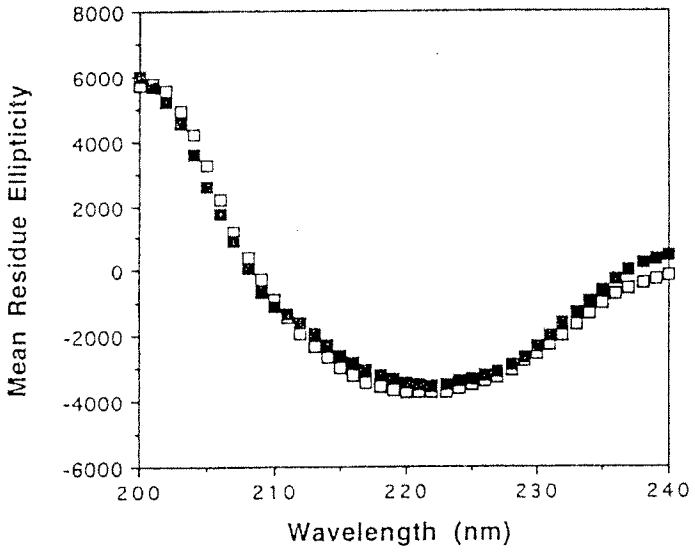


Fig. 1. Far UV-CD spectra of the wild-type TNF (■) and M3 mutant (□).

and Pro-51 on one side and Ala-156 and Pro-12 on the other side. The C-terminal region, including Ile-154 and Ala-156, folds into a β-strand to form the hydrophobic core structure and also mediates intramolecular contacts in a TNF-α trimer. It was suggested that the substitution of Leu-157 at the C-terminus by Phe, Met or Gln increases bioactivity, by increasing tighter hydrophobic intersubunit interactions (14). Substitution of tyrosine by phenylalanine causes a slight decrease in the dimension of the side chain, but produces a significant increase in the hydrophobicity. It was reported that introduction of neutral or charged residues at position 56 significantly reduces the

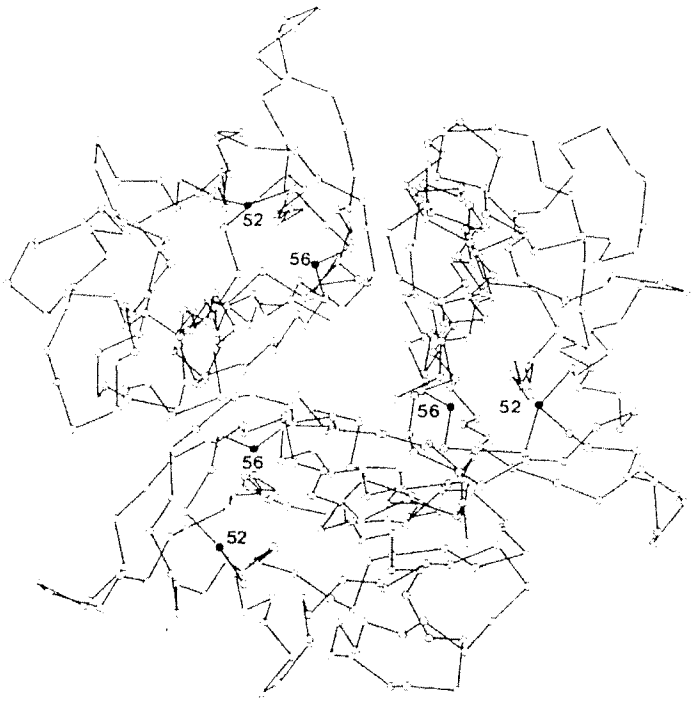


Fig. 2. Bottom view of the TNF molecule indicating the mutation positions (52 and 56) on the TNF trimer.

cytotoxicity of TNF (12). Taken together, these results suggest that phenylalanine residue at position 56 enhances the hydrophobic interaction in this region. And this interaction could be further stabilized by the isoleucine residue at position 52. As shown in the CD data, the overall conformation change by the mutations appears to be negligible, suggesting that the local conformation change in the region 52-56 is mainly responsible for the enhanced cytotoxicity and receptor binding affinity.

The results of present study indicate that the enhanced cytotoxicity coincides with an increase of receptor binding affinity. In the study of TNF derivatives, it has been reported that growth-enhancing activity on FS-4 cells had no correlation with receptor binding (30). Since TNF exhibits many biological activities, it would be interesting to compare other activities of TNF and M3 mutant.

We have produced a mutant TNF-α in which Leu29Ser mutation was added to M3. In contrast to M3, this mutant, referred as M3S, was expressed as a soluble form in *E. coli* (31). In a preliminary study, M3S showed a lower and preferential binding affinity for

TNF-R55, a prolonged half-life *in vivo* and substantially reduced systemic toxicity in mice but higher *in vivo* antitumor activity than wt-TNF-α (Shin, N.-K., Lee, I. And Shin, H.-C., unpublished results). When combined with paclitaxel, M3S was found to potentiate the antitumor efficacy of paclitaxel through the enhancement of apoptosis (32). Thus, it is anticipated that the highly potent M3 mutant can provide a structural basis for a design of optimized TNF-α as a therapeutic purpose.

ACKNOWLEDGMENT

This work was supported by a research grant from the Ministry of Science and Technology (G7) in Korea.

REFERENCES

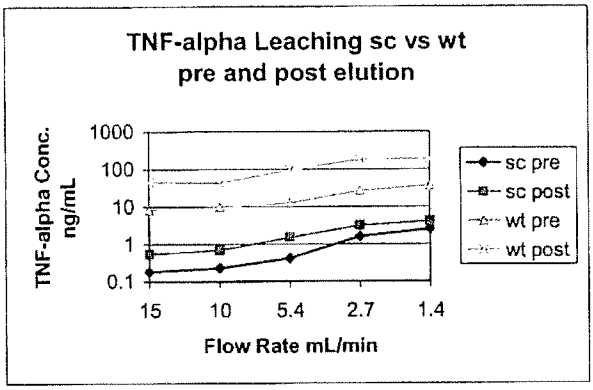
1. Aggarwal, B.B. (1987) Drugs of the Future 12, 891-898.
2. Fiers, W. (1991) FEBS Lett. 285, 199-212.
3. Hauser, G.J., McIntosh, J.K., Travis, W.D. and Rosenberg, S.A. (1990) Cancer Res. 50, 3503-3508.
4. Kilbourm, R.G., Gross, S.S., Jubran, A., Adams, J., Griffith, O.W., Levi, R. and Lodato, R.F. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3629-3632.
5. Van der Poll, T., Van Deventer, S.J.H., Hack, C.E., Wolbink, G.J., Avarden, L.A., Buller, H.R. and Ten Cate, J.W. (1992) Blood 79, 693-698.
6. Ostade, X.V., Vandenabeele, P., Eveeraerdt, B., Loetscher, H., Gents, R., Brockhaus, M., Lesslauer, W., Tavernier, J., Brockaert, P. and Fiers, W. (1993) Nature 361, 266-269.
7. Van Ostade, X., Vandenabeele, P., Tavernier, J. and Fiers, W. (1994) Eur. J. Biochem. 220, 771-779.
8. Loetscher, H., Stueber, D., Banner, D., Mackay, F. and Lesslauer, W. (1993) J. Biol. Chem. 268, 26350-26357.
9. Lienard, D., Lejeune, F., Delmotte, J.-J., Renard, N. and Ewalenko, P. (1992) J. Clin. Oncol. 10, 52-60.
10. Tartaglia, L.A., Weber, R.F., Figari, I.S., Reymonds, C., Palladino, Jr., M.A. and Goeddel, D.V. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 9292-9296.
11. Loetscher, H., Steinmetz, M. and Lesslauer, W. (1991) Cancer Cells 3, 221-226.
12. Zhang, X.-M., Weber, I. and Chen, M.-J. (1992) J. Biol. Chem. 267, 24069-24075.
13. Van Ostade, X., Tavernier, J., Prange, T. and Fiers, W. (1991) EMBO J. 10, 827-836
14. Van Ostade, X., Tavernier, J. and Fiers, W. (1994) Protein Engng. 7, 5-22.
15. Creasey, A.A., Doyle, L.V., Reynolds, M.T., Jung, T., Lin, L.S. and Vitt, C.R. (1987) Cancer Res. 47, 145-149.
16. Kamijo, R., Takeda, K., Nagumo, M., Konno, K., Hasegawa, A., Inaka, K. and Ikehara, M. (1989) Biochem. Biophys. Res. Commun. 160, 820-827.
17. Soma, G.-I., Kitahara, N., Tsuji, Y., Kato, M., Oshima, H., Gatanaga, T., Inagawa, H., Noguchi, K., Tanabe, Y. and Mizuno, D. (1987) Biochem. Biophys. Res. Commun. 148, 629-635.

18. Gatanaga, T., Noguchi, K., Tanabe, Y., Inagawa, H., Soma, G.-I. And Mizuno, D. (1989) J. Biol. Response Mod. 8, 278-286.
19. Masegi, T., Nakamura, S., Fukuoka, M., Kitai, K., Ichikawa, Y., Watanabe, N. and Niitsu, Y. (1993) Biotech. Lett. 15, 1107-1110.
20. Nakamura, S., Kato, A., Masegi, T., Fukuoka, M., Kitai, K., Ogawa, H., Ichikawa, Y., Maeda, M., Watanabe, N. and Niitsu, Y. (1991) Int. J. Cancer 48, 744-748.
21. Taniyama, M., Morita, T., Yamagishi, Y., Kato, A., Bando, C., Okawa, N. and Kaji, A. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 3324-3329.
22. Hanahan, D. (1983) J. Mol. Biol. 166, 557-580.
23. Mosmann, T. (1983) J. Immunol. Methods 65, 55-63.
24. Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. and Williamson, B. (1975) Proc. Natl. Acad. Sci. USA 72, 3666-3670.
25. Brockhaus, M., Schoenfeld, H.-J., Schlaeger, H.-J., Hunziker, W., Lesslauer, W. and Loetscher, H. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3127-3131.
26. Tartaglia, L.A. and Goeddel, D.V. (1992) Immunol. Today 13, 151-153.
27. Jones, E.Y., Stuart, D.I. and Walker, N.P.C. (1989) Nature, 338, 225-228.
28. Eck, M.J. and Sprang, S.R. (1989) J. Biol. Chem. 264, 17595-17605.
29. Smith, R.A. and Baglioni, C. (1987) J. Biol. Chem. 262, 6951-6954.
30. Tsujimoto, M., Tanaka, S., Sakuragawa, Y., Tsuruoka, N., Funakoshi, K., Butsugan, T., Nakazato, H., Nishihara, T., Noguchi, T. and Vilcek, J. (1987) J. Biochem. (Tokyo) 101, 919-925.
31. Jeong, W., Shin, N.-K. and Shin, H.-C. (1997) Biotechnol. Lett. 19, 579-582.
32. Seong, J. Milross, C.G., Hunter, N.R., Shin, H.-C. and Milas, L. (1997) Anti-Cancer Drugs 8, 80-87.

Results of Assays 20080618 and 20080619:

Flow Rate	Single Chain TNF-α ng/mL		WT Recomb. TNF-α ng/mL		Leach Rate wt post		Leach Rate sc post		Leach Ratio
mL/min	sc pre	sc post	wt pre	wt post	ng/min	ug/hr*	ng/min	ug/hr*	wt/sc
15	0.182	0.541	8.2	44.3	664.50	159.48	8.12	1.95	81.89
10	0.227	0.689	9.8	41.9	419.00	100.56	6.89	1.65	60.81
5.4	0.415	1.524	12.3	95	513.00	123.12	8.23	1.98	62.34
2.7	1.609	3.138	26.2	178	480.60	115.34	8.47	2.03	56.72
1.4	2.52	4.151	35.7	184.1	257.74	61.86	5.81	1.39	44.35
					AVE	112.07	AVE	1.80	61.22
					SD	35.48	SD	0.27	13.54

*Normalized to human size device



	Average scTNF-α Leach Rate µg/hr	Leach Rate Ratio of TNF-α to scTNFa µg/hr
Average TNF-α Leach Rate µg/hr		
112.07 ± 35.48	1.8 ± 0.27	62.3 ± 13.54

	TNF-alpha	scTNF-alpha
Leach Rate mcg/hr	112.07	1.8
StDev	35.48	0.27